(1) Title: Effects of Silver and Other Metals on the Cytoskeleton

(2) Type of Report: Summary of Research [= the "Final Technical Report"]
The text below will be accompanied by Form SF-298.

(3) Principal Investigator: Gary W. Conrad

(4) Period covered by the report: 02/15/95 - 06/30/97

Note: The original notice of grant award was for a 3 yr grant from 02/15/95-02/14/98 as grant No. NAGW-4491 (copy of award notice is enclosed). For reasons never clear to me or to my business office, that grant was declared terminated at the end of 2 years]. Perhaps the termination occurred in association with transfer of budget authority from NASA Headquarters to the Department of the Navy. My business office and I did not learn of this transfer until well into 1997, thus accounting for our need to ask for an extension (it was extended until 06/30/97, which we greatly appreciated). In addition, I did not understand that the originally awarded grant would actually "terminate" on 06/30/97, (and so did not file a "Final Technical Report"), because I reasoned that the project was continuing for the next year (beginning 02/15/97) as NAG5-3885.

Compounding this confusion has been my confusion over the types of reports due and their expected receipt dates. My records indicate that I filed all requested Data Update Forms for FY95, FY96, and FY97, and the annual performance reports for the first and second years of the grant. However, this year I became busier than usual with end-of-semester teaching responsibilities and forgot to submit such a report for the past year. Thus, I apologize for my oversight in missing the Dec. 14 deadline for submitting the annual performance report for NAG5-3885. I will submit it as soon as possible - before Jan. 14.

(5) Recipient institution:

Dr. Ronald W.Trewyn
Associate Vice Provost for Research
Kansas State University
Office of Research and Sponsored Programs
2 Fairchild Hall
Manhattan, KS 66506-1103

(6) Grant No. NAGW-4491

Final Technical Report

(1) Comparison of actual accomplishments with the goals and objectives for this period:

a. Determine the role of sulfhydryl groups in cell response to silver ions (Ag^+): Heavy metal ions, such as Ag^+, are thought to affect cellular functions by binding to proteins via a cage of three (3) sulfhydryl groups (-SH). We asked if fertilized eggs of

our model organism, *Ilyanassa obsoleta* (marine mudsnail) would show responses that mimiced those to Ag^+ if -SH groups were cross-linked instead in groups of two (2) by homobifunctional-sulfhydryl-specific cross-linking reagents - all in the absence of Ag^+. One such agent was identified that produced effects on cellular shape change that mimiced the effects of Ag^+. These data suggest that, by whatever mechanism Ag^+ is affecting these cells, the mechanism involves -SH groups.

In support of this conclusion, we determined the extent to which Ag^+ could exert its effects in the presence of either reducing agents or oxidizing agents. Results indicated that in the presence of a reducing agent, dithiothreitol (DTT), the effects of Ag^+ are blocked, whereas in the presence of an oxidizing agent, H_2O_2, the effects of Ag^+ are not blocked.

b. Determine if other heavy metal ions affect the cellular shape changes of the fertilized *Ilyanassa* eggs:

We have determined that gadolinium ion (Gd^{3+}) does not cause the same effects on cellular shape change as does Ag^+. In contrast, a narrow range of both copper ions (Cu^{2+}) and gold ions (Au^{3+}) cause the formation of a long stabilized neck of cytoplasm by fertilized eggs undegoing first cleavage, a pattern resembling that of Ag^+.

c. Determine whether Ag^+ exerts its effects by entering cells and determine its site of action in causing long stabilized necks of cytoplasm to form containing many microtubules.

Ionophores are substances that allow charged molecules to cross cell membranes. The work of Tsukube et al. (1995. Tetrahedron Lett. 36: 257-2260) described "ionophores exhibiting perfect Ag^+ ion selectivity." We obtained samples of these compounds from Dr.Tsukube and applied them to fertilized eggs of Ilyanassa in sea water in the presence and absence of Ag^+ to determine if the cellular sensitivity to Ag^+ was increased in the presence of such agents that should increase the intracellular concentration of Ag^+. Results indicated that none of the three ionophores tested changed the cell sensitivity to Ag^+.

Myosin is a contractile protein component of the cytoskeleton of cells, an active participant in many cellular shape changes by virtue of its rod-like structure for polymerizing into a variety of forms (e.g., thick-filament-like arrays of muscle) and because its head region interacts with F-actin and generates mechanical force by changing shape (as a "mechanoenzyme") as an ATPase site, that is also in the head region, hydrolyzes ATP. Cramer and Mitchison (1995. J.Cell Biol. 131: 179-189) described the effects of BDM on cellular shape changes; BDM is an inhibitor of the ATPase activity of myosin ATPases, of both muscle and non-muscle origin. Result: BDM elicited abnormal cell shape changes in fertilized *Ilyanassa* eggs that greatly resembled those seen in response to Ag^+. Thus, Ag^+ may inhibit normal cellular shape changes by inhibiting the myosin-ATPase (s) that participate in those shape changes.

To determine if *Ilyanassa* myosin is involved in the cellular shape changes observed in fertilized eggs, and to determine if Ag^+ inhibits these normal shape changes by interacting with the ATPase of myosin, antibodies will be required for cytolocalization of the myosin. Thus, we began by using techniques of molecular biology to obtain the cDNA for *Ilyanassa* myosin and determined the sequence of the N-terminal

region of the myosin heavy chains that includes the actin-binding domain and the ATPase site. That information will be translated as amino acid sequence to allow the chemical synthesis of corresponding peptides that can be injected into rabbits (commercially) for production of antisera against *Ilyanassa* myosin(s). Such antisera will be used for cytolocalization of myosin in fertilized eggs during the shape changes. Using the same sources of RNA and the same molecular techniques, partial sequences are also being obtained for the cDNAs for other cytoskeletal proteins and factors that regulate heart muscle development.

(2) Reasons why established goals were not met:

Three of the originally proposed objectives require that we be able to immobilize the fertilized *Ilyanassa* eggs so that we can: a) determine the effect on development of altered orientations of the cells with respect to gravity (they normally develop with their "heavy" end down), b) determine if their response to an altered orientation to gravity is enhanced in the presence of Ag^+, and c) determine if development is altered in such immobilized cells if they are subjected to externally-applied constant compressive stress or constant tensile stress. No techniques of cellular immobilization so far tested have allowed holding the cells in a pre-determined position without also killing them. Nevertheless, new techniques for immobilizing cells are being described, so it is likely that one of them will be applicable to these fertilized eggs. This is important to persue, because these fertilized *Ilyanassa* eggs remain the most sensitive type of animal cell (vertebrate or invertebrate) for displaying the effects of Ag^+ on cytoskeleton-dependent phenomena.

(3) Plans for the next year of research on this project:

As explained on page 1, this project is continuing under the NASA grant No. NAG5-3885. As will be described in the annual progress report of that grant, we have continued to do experiments to: a) determine the subcellular site of action of Ag^+ on this cytoskeleton-dependent cellular shape change, b) obtain more cDNA sequence of the most likely major proteins involved in cytoskeleton action and in early differentiation of the cytoskeleton and such tissues as the heart (an organ that fails to differentiate in the presence of Ag^+ because of the Ag^+-induced loss of a critical portion of cytoplasm from the fertilized egg [called the polar lobe]), and c) devise a technique for immobilizing these cells without killing them.

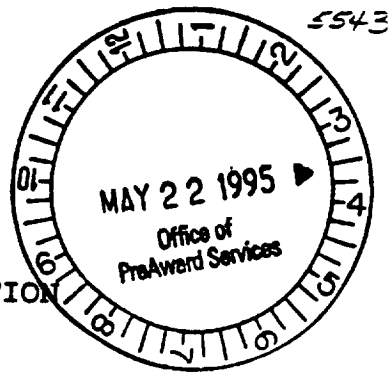
Respectfully submitted,

Gary W. Conrad

Gary W. Conrad Professor and Principal Investigator

Enclosures: Original notice of grant award for 3 yrs (02/15/95-02/14/98)
Authorization for no-cost extension of NAGW-4491 to 06/30/97
Letter of 12/10/97 from Normilita Poblete re Final Tech. Report
Published abstracts from Bull.Mount Desert Island Biol.Lab:
34: 4-5 (1995); 34: 103-104 (1995)
35: 5-6 (1996): 35: 17-18 (1996)

5543



NAGW-4491
Supp. Basic

RESEARCH GRANT AWARD
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION
Washington, DC

This Grant is awarded to support research pursuant to P.L. 97-258 (31 U.S.C. 6301, et seq.) and will be administered in accordance with the "NASA Provisions for Research Grants and Cooperative Agreements," attached hereto and incorporated herein, and in conformity with any appended "Special Conditions" or other written understandings between NASA and the Grantee relating to this Grant.

1) No.: **NAGW-4491**
Supplement: Basic

2) Amount Awarded:
$383,340

3) For a period of approximately thirty-six (36) months beginning about February 15, 1995.

4) To: Kansas State University
Attn: Mr. James Shanteau
Vice Provost for Research
2 Fairchild Hall
Manhattan, KS 66506-1103

5) For Research Entitled: "Effects of Silver and Other Metals on the Cytoskeleton"

6) Under the Direction of: Gary W. Conrad

7) The NASA Technical Officer for this Grant is:
Vicki Thorne, Code: UL, Phone: (202) 358-2339

8) UNITED STATES OF AMERICA

Adriene Woodin 5/16/95
(Signature) (Date)
Adriene Woodin
Contracting Officer

==========

FUNDING HISTORY
Total Amount Awarded: $383,340
Previous Obligations: $ -0-
Current Obligations: $122,803
Total Oblig. to Date: $122,803

NASA DATA
Proc. Request No.: 10-80273
Appropriation: 29 805/60110 2512
199-40 ULA00 10-01-04
PPC Number: RT

Property/Close-out Administration (if applicable) by: ONRRR/4520 Executive Drive, Suite 300, San Diego, CA 92121-3019.

NASA Grants Negotiator: Elaine J. Powell
Phone: (202) 358-0574
EPowell@PROC.HQ.NASA.GOV
FAX: (202) 358-3899

Imp - Authoriz of extension of 5-30620 until June 30, 1997

KSU
KANSAS STATE UNIVERSITY

Office of Research and Sponsored Programs

PreAward Services
2 Fairchild Hall
Manhattan, Kansas 66506–1103
913-532-6804
FAX: 913-532-5944

Extension of 5-30620 (Authoriz. of) thru June 30 1997

February 21, 1997

Adriene Woodin, Grants Officer
National Aeronautics and Space Administration
Headquarters
Washington, DC 20546-0001

RE: NASA Grant No. NAGW-4491
Principal Investigator - Gary W. Conrad

This letter is notification that under Organizational Prior Approval System (OPAS), I have, effective February 21, 1997, approved a no-cost four and one-half-month extension of the subject grant through June 30, 1997. The objectives of this grant will more likely be accomplished with such an extension.

Sincerely,

R. W. Trewyn

R. W. Trewyn
Associate Vice Provost for Research
OPAS Officer

RWT/dec

cc: Vicki Thorne, Technical Official
Gary Conrad ✓
Brian Spooner

Department of the Navy
Office of Naval Research
San Diego Regional Office
4520 Executive Drive Suite 300
San Diego, CA 92121-3019

Controller's Office

DEC 17 1997

Sponsored Project Accounting

In Reply Refer to:
4330:246:cgl
NAGW-4491
10 December 1997 5-30620

Ms. Patsy Havenstein
KANSAS STATE UNIVERSITY
Kansas State University
10 Anderson Hall
Manhattan, KS 66506 0108

Attn: Contract and Grant Closeout Administrator/Coordinator:

The grant number listed below has expired. In order to close out this agreement and process final payment, the following documents identified as REQUIRED need to be submitted within 60 days.

AGREEMENT NUMBER:	PRINCIPAL INVESTIGATOR:	EXPIRATION DATE:
NAGW-4491	Mr. Gary Conrad	6/30/97

FINAL TECHNICAL REPORT: *REQUIRED*
Final technical report, performance report, or other deliverable and transmittal document for the report. Documentation furnished must show the date the report was submitted to the Project Officer or Program Manager and include a copy of the Standard Form 298 (Report Documentation Page) for DOD programs or as required by the agreement terms and conditions. A completed Standard Form 298 must be completed and submitted to the Defense Technical Information Center (DTIC) or other location in accordance with the terms of your agreement. (For your convenience, a blank SF-298 is attached for your use)

FINAL VOUCHER/CASH TRANSACTIONS REPORT: *REQUIRED*
Final Voucher, for Contracts, including the Contractor's Release of the Government and Contractor's Assignment of Refunds, Rebates and Credits. If this agreement is a GRANT, a Final Financial Status Report (SF-269) and summary of GRANT costs shall be submitted in lieu of a final voucher. (See your agreement for specific requirements)

FINAL PROPERTY/INVENTORY REPORT: *RECEIVED*
Final Property Inventory requesting disposition instructions. Provide separate listings for Government Furnished and Contractor Acquired property. Once disposition instructions are provided by this office, a final DoD DD-1662 or NASA 1018 is required in order to finalize all property/inventory actions for government owned items.

FINAL PATENT/INVENTION REPORT: *RECEIVED*
Final Patent/Invention Disclosure Report or other documentation required by the agreement Patents Clause. (Usually DD-Form 882).

FINAL SECURITY REPORT: *NOT REQUIRED*
Certification that disposal of classified material is complete. (Contracts containing a DD-254)

FINAL NEW TECHNOLOGY REPORT: *NOT REQUIRED*
Final NASA New Technology Report (Applies to NASA Contracts only)

Any funds excess to the requirements of this agreement shall be promptly reported to the government contract/grant administrator. If you have any questions concerning any closeout requirements, please contact me at (619) 677-6464. The ONR office FAX number is (619) 677-6480.

Sincerely

MS. NORMILITA POBLETE
Procurement Technician

If this agreement is being renewed/extended, please complete the information indicated below and return a copy of this letter to our office. Please include a copy of your proposal forwarding letter. THANK-YOU

Proposal Number/ID:	Date Submitted:	Requested Start Date:

Bulletin of the Mount Desert Island Biological Laboratory. Vol. 34 (1995)

THE EFFECTS OF HEAVY METALS ON CYTOSKELETAL COMPONENTS INVOLVED IN CELL SHAPE CHANGES DURING FIRST CLEAVAGE IN ILYANASSA OBSOLETA

A.H. Conrad, M.J. Janasek, S.S. Schwarting, & G.W. Conrad
Division of Biology, Kansas State University, Manhattan, KS 66506-4901

During first cleavage in the marine mollusk, Ilyanassa obsoleta, two contractile rings are formed at right angles to each other in the fertilized egg. The cleavage furrow (CF) forms at the animal pole of the egg, equidistant between the two mitotic asters, and constricts across and around the animal hemisphere of the egg, in a plane perpendicular to the mitotic spindle axis, to form an intercellular bridge containing microfilaments (MFs), composed of F-actin, encircling midbody microtubules (MTs). The CF remains permanently constricted and eventually cleaves the two daughter cells apart from one another. The polar lobe constriction (PLC) forms in the vegetal hemisphere, beneath the mitotic apparatus, and constricts around the vegetal hemisphere of the egg, in a plane parallel to the mitotic spindle axis, to form a very tight polar lobe neck eventually containing no MFs and encircling no MTs. Normally, the PLC is a transient constriction and eventually relaxes, such that the polar lobe vegetal cytoplasm merges with one of the two daughter cells that formed from the animal hemisphere. In the presence of 5-7 X 10^{-11} M Ag^+, generated from diluting saturated solutions of $AgNO_3$ in sea water, the usually transient PLC becomes very elongated, encircles many MTs, becomes stably constricted, and often results in permanent separation of the polar lobe from the animal hemisphere cell to which it was attached (Conrad, AH, et al. 1994. Cell Motil. Cytoskel.27:117-132).

In order to demonstrate that it is the Ag^+ ions that cause the PLC stabilization, and not some other ion present as a contaminant in the $AgNO_3$ sea water solutions, silver wires (99.99% Ag^0) were used as (+) and (-) electrodes in separate electrode baths connected by an agarose bridge prepared in Millipore-filtered (0.45 μm) sea water (MFSW) to generate Ag^+ in the anode MFSW bath by electrolysis. After electrolysis, each electrode solution was adjusted to pH 7.7, as necessary, then diluted with more MFSW, and used to incubate fertilized eggs. The anode solution, containing free Ag^+, generated elongated stabilized PLCs, whereas the cathode solution did not alter PLC dynamics, compared to control eggs. In addition, gold, present as 3.5 μm $AuCl_3$ in MFSW, caused elongation and stabilization of the PLC during first cleavage, in a manner similar to that of Ag^+, whereas gadolinium, present at 1 mM $GdCl_3$ in MFSW, had no effect on cleavage or polar lobe formation.

In the previous silver study, it was postulated (a) that the presence of MTs in the PLC at maximum constriction allows the PLC contractile ring to retain MFs, as does the CF at maximum constriction when it encircles midbody MTs, and (b) that the retained MFs stabilize the constriction of the normally transient PLC. To test this hypothesis, Ilyanassa obsoleta fertilized eggs were allowed to develop in 5.2 X 10^{-11} M Ag^+-MFSW to the time of maximum CF/PLC constriction, then extracted, fixed, and triple-stained with antibodies to α-tubulin (localized with TRITC-labeled secondary antibody) to visualize MTs, FITC-labeled phalloidin to visualize F-actin, and Hoechst 33258 to visualize DNA. Control eggs showed F-actin in the CF and across the top of the polar lobe and into the PLC during early cleavage and PL neck constriction. However, at the time of maximum constriction, F-actin was still visible in the intercellular bridge of the CF around the midbody MTs, but no F-actin nor MTs were visible in the PLC. In

contrast, in the presence of Ag^+, both F-actin and MTs were visible in the CF and in the elongated PLC at the time of maximum constriction. We conclude that Ag^+-treated cells not only show increased numbers of MTs, but, judging from the immunofluorescence data, may show increased arrays of MFs as well. The latter conclusion must be confirmed by electron microscopy. Work supported by NASA-BioServe NAGW-1197 and NASA-NSCORT NAGW-2328.

Bulletin of the Mount Desert Island Biological Laboratory. Vol. 34 (1995)

NATRIURETIC PEPTIDE EXPRESSION IN ILYANASSA OBSOLETA

A.H. Conrad, S.S. Schwarting, and G.W. Conrad
Division of Biology, Kansas State University, Manhattan, KS 66506-4901

Mammals synthesize a family of highly conserved small natriuretic peptides that modulate fluid and electrolyte homeostasis and that are expressed in tissue-specific patterns: (a) ANP: a 28 aa polypeptide originally detected primarily in cardiac atria and ventricles during embryonic development and in cardiac atria in adults (Zeller, R. et al., 1987, GenesDev.1:693-698); also detected in very low amounts in the central nervous system, thyroid, lung, kidney, spleen, thymus, ovary and uterus; but not in striated muscle cells (Vollmar, A.M. 1990, Klin.Wochenschr.68:699-708); (b) BNP: a 26-45 aa polypeptide expressed mainly in the heart, but also detected in the central nervous system, lung, thyroid, adrenal, kidney, spleen, small intestine, striated muscle, ovary, and uterus (Gerbes, A.L. et al., 1994, J.Clin.Endocrin.Met.78:1307-1311); and (c) CNP: a 22 aa polypeptide detected only in the central nervous system (Komatsu, Y. et al., 1991, Endocrinology1 29:1104-1106), endothelium (Suga, S. et al., 1992, J.Clin.Invest.90:1145-1149), and monocytic cells (Ishizaka, Y., et al., 1992, Biochem.Biophsy.Res. Commun.189:697-704). In other organisms, the presence and tissue distribution of natriuretic peptides are less well understood. In frogs, the principal vasorelaxant peptide is CNP, present in various isoforms (Yoshihara, A. et al., 1990, Biochem.Biophys. Res.Commun.173:591-598). The dogfish shark heart expresses CNP (Schofield, J.P. et al., 1991, Am.J.Physiol. 261:F734-F739), and some fish express BNP-like and/or CNP-like, but no ANP-like, immunoreactivity in heart and brain tissue (Donald, J.A. et al., 1992, MDIBL Bull.31:120-121). Eels have ANP, CNP, and a ventricle-specific VNP, but no BNP (Takei, Y. et al., 1991, FEBSLett.282:317-320). Using a polyclonal antibody that is ANP-specific in mammalian natriuretic peptide cross-reactions, ANP has been detected immunochemically in the eggs of both veterbrates(Kim, S.H. et al., 1993, Comp. Biochem.Physiol.104A:219-223) and invertebrates (Kim, S.H. et al., 1994, Gen.Comp.Endocrinol.94:151-156), as well as in hearts of the oyster (Vesely, D.L. et al., 1993, Comp.Biochem.Physiol.106B:535-546) and the earthworm (Vesely, D.L. et al., 1992, Comp.Biochem.Physiol.101C:325-329), in paramecium (Vesely, D.L. et al., 1992, Peptides13:177-182), and in the roots, stems, leaves, and flower petals of higher plants (Vesely D.L. et al., 1993, Am.J.Physiol.265:E465-E477). The adult marine mollusc Ilyanassa obsoleta contains a two chambered heart, but if the third polar lobe cytoplasm is removed from the egg at first cleavage, the heart and several other tissues fail to form. We have used immunocytochemical and PCR methods to examine the tissue specificity of ANP and CNP expression in adult snails and in eggs, third polar lobes, and Day 5 and Day 6 (D5 & D6) lobed and lobeless Ilyanassa embryos.

The precleavage eggs from 10 capsules and samples of adult heart auricle, heart ventricle, gill, kidney, intestine, mantle, and ovary were collected separately into TBSA (Tris buffered saline, pH 6.8, with 0.1% NaN_3 [Sodium Azide]) containing 10 mM EDTA (Etylenediaminetetraacetic Acid) and 25 μM PMSF (Phenylmethylsulfonyl Fluoride) on ice, homogenized, microfuged to remove insoluble cellular material, boiled for 5 min to inactivate endogenous alkaline phosphatase and other proteases, and spotted onto nitrocellulose paper. Dot blots were blocked with Blotto, developed with antibody to human/canine ANP that does not react with human BNP or porcine CNP (Peninsula Laboratories, Inc.), and visualized with alkaline-phosphatase-labeled secondary antibody. All tissues

showed some reactivity, but the auricles, gills, mantle and ovary were the most responsive on a per unit soluble protein basis. Control blots developed without primary antibody showed no staining at all, while those developed with anti-alpha tubulin showed a tissue distribution different from that of the natriuretic peptide distribution. These results suggest that marine snails express natriuretic peptide(s) in many tissues.

However, the isoform specificity of the antibody has not been confirmed in nonmammalian systems, so RT-3' RACE (Rapid Amplification of cDNA Ends; Gibco-BRL)-PCR using nested degenerate 5' primers specific for ANP and CNP, respectively, was carried out in order to determine whether multiple natriuretic peptide isoforms are present in marine snail tissues. Tissues were isolated in Millipore-filtered (0.22 μM) sea water, mRNA was isolated by the Micro-Fastrack procedure (Invitrogen), cDNA was synthesized using 3'-RACE procedures, and the cDNA was amplified using one of the 5' natriuretic primers at 52°C. Auricles, ventricles, gills, and mantle, but not the ovary, precleavage eggs, or D5 or D6 lobed or lobeless embryos, yielded a band of ~ 680 bp with the most N-terminal ANP primer, and ventricles gave a band of ~ 550 bp both with the more C-terminal ANP primer and with the more C-terminal CNP primer that contained some overlapping sequence with the C-terminal ANP primer. Auricles, ventricles, kidney, mantle, intestine, eggs before first cleavage, and isolated third polar lobes yielded a 920 bp band with the most N-terminal CNP primer. With the more C-terminal CNP primer (derived from the very highly conserved, CNP-specific, KLDRIG sequence) adult tissues plus the ovary, D5 lobed and lobeless embryos, and D6 lobed embryos yielded an 840 bp band and a 470 bp band, whereas the D6 lobeless embryos yielded only the 470 bp band. The auricle CNP-N-terminal 920 bp band yielded an 840 bp band when re-expanded with the nested CNP-C-terminal primer. The ubiquitous KLDRIG 470 bp band has been sequenced and found to be the widely conserved ribosomal protein L27a. Sequencing of the ventricular ANP/CNP 550 band and the more wide-spread CNP 920/840 bands are in progress. These preliminary studies suggest that Ilyanassa obsoleta may express more than one isoform of natriuretic peptide, and that one of them may be ventricle-specific. This work was supported by NASA-BioServe NAGW-1197 AND NASA-NSCORT NAGW-2328.

Bulletin of the Mount Desert Island Biological Laboratory. Vol. 35 (1996)

MECHANISMS OF SILVER ION (Ag^+) TOXICITY IN FERTILIZED EGGS OF ILYANASSA OBSOLETA

G.W.Conrad[1], M.J.Janasek[1], N.M.Martinez[2], and A.H.Conrad[1]
[1]Division of Biology, Kansas State University, Manhattan, KS
[2]Department of Biology, Georgia Southern University, Statesboro, GA

We have demonstrated previously that microtubule distribution in fertilized eggs of the common marine mudsnail, Ilyanassa obsoleta Stimpson (=Nassarius obsoletus Say) is very sensitive to the presence of silver ions (Ag^+) in the sea water (A. Conrad, et al. Cell Motil. & Cytoskel. 27: 117-132 (1994)): a narrow range of Ag^+ concentrations (5-7 x 10^{-11}M) causes a marked increase in the numbers of microtubules in a normally transient cytoplasmic neck (polar lobe constriction), followed by great elongation of the neck and its eventual severing, an abnormal developmental event. This response largely mimics the cellular response to the reference standard microtubule stabilizing agents, taxol and hexylene glycol (A. Conrad et al. J. Exp. Zool. 262: 154-165 (1992) and J. Exp. Zool. 269: 188-204 (1994)). Heavy metal ions, such as Ag^+, are thought to interact with proteins via a cage formed from three sulfhydryl groups. We therefore asked if any other metal ion could duplicate the Ag^+ response and whether it could be mimicked by agents that could form cross-links between sulfhydryl groups, in the absence of Ag^+.

Effects of other metal ions: In a narrow range of concentrations (0.75-1.5 μM), we observed that Cu^{2+} causes 13- 37% of Ilyanassa fertilized eggs to form very long polar lobe necks resembling those formed in response to Ag^+. This indicates that Ag^+ is not the only heavy metal ion to cause this effect.

Effects of cross-linking reagents: Four homobifunctional sulfhydryl cross-linking reagents were assessed for their ability to elicit Ag^+-like cellular deformation of fertilized Ilyanassa eggs (very long, thin polar lobe necks; a shape not seen during normal development): N,N'-p-phenylene dimaleimide (p-PDM), N,N'-bis(3-maleimidopropionyl)- 2-hydroxyl-1,3-propanediamine (N,N'-bis), Bismaleimidohexane (BMH), and 1,4-Di-[3'-(2'pyridyldithio)propionamido]butane (DPDPB). When applied to cells in sea water, p-PDM, N,N'-bis, and BMH either caused no cells to form abnormally elongated polar lobe necks, or caused very few to form (N,N'-bis; <5% of cells). In contrast, exposure of cells to DPDPB caused as many as 47% of cells to assume this unusual shape. The range, 75-250 μM, causes an average of 6 % or more cells to form long necks, with the optimum concentration of 125 μM causing an average of 11% with long necks. If eggs are pretreated with a reducing agent, dithiothreitol, followed by DPDPB, the percentage of responding cells increases to 24%, whereas if the pretreatment is with an oxidizing agent, H_2O_2, the percentage of responding cells is 19 % (continuous treatment with dithiothreitol gives only 3% responding cells, whereas continuous exposure to H_2O_2 gives 4% responding). We conclude that Ag^+-like morphological effects can be produced by treatment with a homobifunctional

sulfhydryl reactive cross-linking reagent. (Support: NASA NAGW-4491, NASA-NSCORT NAGW-2328, & NSF REU 9322221)

Bulletin of the Mount Desert Island Biological Laboratory. Vol. 35 (1996)

MOLECULAR CHARACTERIZATION OF MYOSIN AND THE SODIUM-PROTON ANTIPORTER IN ILYANASSA OBSOLETA

A.H. Conrad & G.W. Conrad
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Ilyanassa obsoleta Stimpson, the common marine mud snail, offers an excellent experimental system for examining possible effects of zero gravity on embryogenesis in general and for determining cytoplasmic factors essential for heart development in particular. Large yolk platelets accumulate gravocentrically in the vegetal pole cytoplasm of the Ilyanassa egg, displacing the nucleus to the animal pole. As a result, Ilyanassa obsoleta embryos sequester their vegetal hemisphere cytoplasm in an anuclear polar lobe at the time of their acentric first cleavage, then merge that polar lobe cytoplasm with just one of the daughter cells, and eventually form their hearts (as well as other tissues) from the cellular descendants of the polar lobe cytoplasm-containing daughter cell. If the polar lobe cytoplasm is divided equally between the two first cleavage daughter cells, morphogenesis, especially of lobe-dependent structures, is disturbed in 40% of the embryos (Render, J., J. Exp. Zool. 253:30-37, 1990). If the polar lobe is removed from the embryo at the time of first cleavage, developing embryos fail to form a beating heart, eyes, statocysts, intestine, operculum, or external shell, although they do form extensive muscle tissue (Atkinson, J.W., J. Morphol. 133:339-352, 1971; Int. J. Invertr. Reprod. Dev. 9:169-178, 1986). To examine the role of cytoplasmic factors in heart muscle development, a rapid, sensitive, and specific assay for Ilyanassa cardiac myocyte differentiation must be developed. Vertebrate heart muscle cells express heart-specific isoforms of the contractile protein myosin-II that can be distinguished from other myosin isoforms by the nucleic acid/amino acid sequence of its amino-terminal globular head region (Bement, W.M., Hasson, T., Wirth, J.A., Cheney, R.E., and Mooseker, M.S. Proc. Natl. Acad. Sci. USA 91:6549-6553, 1994). As a first step in determining whether Ilyanassa obsoleta adult hearts express a heart-specific myosin isoform that may serve as a heart-specific marker for developmental studies, reverse transcriptase-polymerase chain reaction (RT-PCR) studies using degenerate primers for myosin-II were carried out on whole adult Ilyanassa obsoleta. For comparison, a non-heart-specific Ilyanassa probe was developed using degenerate primers for a sodium-proton antiporter.

Total cell RNA was isolated from a whole adult snail using the Promega RNAgents Total RNA Isolation System. Analysis of the RNA on an agarose/ formaldehyde gel revealed intact ribosomal RNA. Degenerate probes for myosin-II were prepared against the 5′ ATP binding site (5′-GARTCNGGNGCNGGNAARAC-3′) and a 3′ site of unknown function (5′-RTGRTTRAANARYTGYTG-3′), based on amino acid sequences conserved from C. elegans to rat, and synthesized by Genemed Biotechnologies, Inc., South San Francisco, CA 94080. Degenerate 5′ and 3′ oligonucleotide probes for the crab sodium-proton antiporter were generously supplied by Dr. David Towle. Ilyanassa

RNA was converted to cDNA using the GibcoBRL Superscript Preamplification System for First Strand cDNA Synthesis, and PCR was subsequently conducted using the GibcoBRL PCR Reagent System with 40 amplification cycles of 94° for 45 sec, 42° for 30 sec, and 72° for 90 sec in a Perkin Elmer Thermocycler. The myosin-II probes yielded an expected band of approximately 925 bp, whereas the sodium-proton antiporter probes yielded an expected band of 710 bp, when separated on agarose gels. These bands were then excised from the gel, cleaned using the Ambion GeniePrep Kit, ligated into gGEMt, transfected into DH5α cloned using blue-white selection, purified by 5 Prime-3 Prime Perfectprep, and sequenced by the KSU Biotechnology Sequencing Center.

Comparison of the Ilyanassa obsoleta myosin cDNA sequence with other known sequences revealed that it is most closely related to Scallop myosin-II, and that it contains sequence identities to both the striated and the smooth muscle myosin isoforms in the critical exon 5-exon 6 region that defines the difference between these two isoforms in the Scallop (Nyitray, L., Jancsó, A., Ochiai, Y., Gráf, L., and Szent-Györgyi, A.G. Proc. Natl. Acad. Sci. USA 91:12686-12690, 1994). The second most closely related myosins are the chicken and rabbit embryonic sarcomeric myosin-IIs, and the third most closely related myosins are the mouse, rat, and human alpha cardiac myosin-IIs. Current studies are underway using degenerate primers to the more 3' myosin actin-binding site to extend knowledge of the Ilyanassa myosin-II sequence to include that region. In addition, other myosin degenerate probes, shown by Blement et al., 1995, to reveal multiple additional myosin class isoforms in human and porcine cells, will be used to investigate the existence of other myosin isoforms in Ilyanassa. Comparison of the Ilyanassa obsoleta sodium-proton antiporter cDNA sequence with other known sequences revealed that it is most closely related to the rat antiporter, followed by the chinese hamster and the crab antiporters. Upon further confirmation, these Ilyanassa sequences will be submitted to Genbank, and will represent the second and third Ilyanassa sequences present in Genbank (the first is for RNA polymerase II: U10338). With this myosin-II sequence known, we are positioned to isolate Ilyanassa adult hearts and to determine, using these and other degenerate myosin probes, whether Ilyanassa hearts make tissue-specific myosin isoforms that may be used for following heart development in lobed and lobeless embryos. Research supported by NASA-NSCORT NAGW-2328.

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13. ABSTRACT ***(Maximum 200 Words)***

Directly or indirectly, trace concentrations of silver ion (Ag^+) stabilize microtubules (Conrad, A.H., et al. 1994. Cell Motil. & Cytoskel. 27:117-132), as does taxol (Conrad, A.H., et al. 1992. J. Exp. Zool. 262:154-165), an effect with major consequences for cellular shape changes and development. Polymerization of microtubules is gravity-sensitive (Tabony and Job, 1992. Proc. Natl. Acad. Sci. USA 89:6948-6952), so trace amounts of Ag^+ may alter cellular ability to respond to gravity. If Ag electrolysis is used to purify water on NASA space vehicles, plants and animals/astronauts will be exposed continuously to Ag^+, a regimen with unknown cellular and developmental consequences. Fertilized eggs of the marine mudsnail, Ilyanassa obsoleta, are the cells in which the effects of Ag^+ on microtubules were discovered. They distribute visible cytoplasmic contents according to gravity and contain cytoplasmic morphogenetic determinants for heart development. The objectives are to determine if the effects of Ag^+, Au^{3+} (of biosensor relevance), or Gd^{3+} (inhibitor of some stretch-activated ion channels) on the cytoskeleton (in the presence and absence of mechanical loading) will affect cellular responses to gravity.

14. SUBJECT TERMS

Silver ions, Ag, heavy metals, cytoskeleton, embryonic development

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