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# EFFECTS OF INHIBITING TRANSGLUTAMINASE DURING EGG FERTILIZATION AND DEVELOPMENT

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**Summary**: Transglutaminase inhibitor (1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride or L-682777) affected the appearance of the fertilization envelope (FE) and subsequent development in *Sphaerechinus granularis* and *Arbacia punctulata* in a concentration-dependent manner. Abnormalities were first visible in forming FE's at the lowest concentration (0.001 mM) of the inhibitor used. The wrinkled appearance of FE's suggested that the treatment prevented the I-T transition, whereas the finding of numerous denuded cells and empty FE's indicated that the inhibitor acted by rendering the FE's very fragile. Cell division by the 2-cell stage was also affected by this active-site-directed transglutaminase inhibitor. With 0.05 mM of the inhibitor, about 50% of all cells were polynucleated. In view of earlier findings that other transglutaminase inhibitors can produce abnormalities with regard to FE wrinkling and deranged cell division, it can be suggested that different forms of transglutaminases might be involved at various stages in embryonic development. 

The presence of the protein cross-linking enzyme: transglutaminase [protein-glutamine: amine-γ-glutamyltransferase EC 2.3.13] in sea urchin eggs has been known for some time (1, 2). Three electrophoretic forms of the enzyme were identified in *Strongylocentrotus purpuratus*, one of which was purified (3). Transglutaminase activity, measured by the incorporation of radioactive putrescine into endogenous protein substrates of the enzyme in the egg, increases dramatically upon fertilization even when protein synthesis is inhibited by emetine (4). With dansylcadaverine, a number of amine-acceptor protein substrates could be labelled during embryonic development (1, 5). The most convincing evidence for the *in vivo* functioning of transglutaminase was provided by the isolation $N^{\varepsilon}$-(γ-glutamyl)lysine crosslinks in significant quantities (2).

The exogenous primary amines (putrescine, dansylcadaverine) act as competitive inhibitors of protein cross-linking reactions catalyzed by transglutaminase. Similarly functioning compounds [such as glycine ethylester, penicillamine, isoniazide,

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benzylhydrazine (6, 7)] were found to interfere with the "stabilization" of the fertilization envelope (FE). Examining the hierarchy of cross-linking reactions in the extracellular matrix, Battaglia and Shapiro (8) demonstrated that an egg surface transglutaminase was involved in the early stages of assembly of the FE. The enzyme was activated by $Ca^{2+}$ and inhibited by $Zn^{2+}$. Radioactive putrescine, similarly by to the findings previously reported for dansylcadaverine (1), became incorporated into the FE during fertilization. Moreover, eggs fertilized in the presence of putrescine or cadaverine gave rise to disorganized and expanded FE's, with a morphological change of casts (8, 9).

The main purpose of the present work was to examine the effects of the recently synthesized (10, 11) 2-[(2-oxopropyl)-thio]imidazolium type of active-site directed inhibitor of transglutaminase on the fertilization of *Spaerechinus granularis* eggs.

## Materials and Methods

The transglutaminase inhibitor: 1,3,4,5-tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride or L-682777 was kindly provided by Dr. Andrew M. Stern of Merck Research Laboratories, West Point, PA. A stock solution of this compound (50 mM) was made up in $Me_2SO$. The final concentration of the inhibitor in the sea water used for fertilizing eggs and culturing embryos ranged between 0.001 and 0.05 mM. Thus the concentration of $Me_2SO$ in the medium did not exceed 0.1% which, as examined in control experiments, did not seem to have any effect on the degree of fertilization, the appearance of FE or on subsequent development. *S. granularis* was collected in the Bay of Naples. Methods for gamete preparation and the fertilization of eggs were essentially the same as described by Cariello et al.(4). Eggs were removed from the body cavity of females and were washed 3 times in filtered sea water (800 ml), each time with passage through four layers of cheese cloth. Prior to allowing the eggs to settle in the final wash fluid, an aliquot was placed on a 25-mm HA 0.45 μm Millipore filter for cell counting. To collect sperm, a male specimen was opened, and the sperms were left in the body cavity until needed, at which time a drop of the concentrated seminal fluid was diluted about one hundred fold with filtered sea water. An egg suspension (5 ml), containing about 5000 eggs/ml, was incubated at room temperature for 10 min with the inhibitor and was then fertilized by adding a drop of diluted sperm. Control cultures were raised in a similar fashion, both with and without inclusion of $Me_2SO$ at the concentration prevailing in the experimental samples. The efficacy of fertilization (i.e. percentage of FE formation), the appearance of FE and further development was assessed with the aid of an Axiophot Zeiss microscope, equipped with Plan-Neuflor (10x, 20x) lens. In each experiment, at least three hundred eggs were examined.

## Results and Discussion

Visible abnormalities in forming FE and in development by the 2- and 4-cell-stages were evident even at the lowest concentration (0.001 mM) of the active-site directed transglutaminase inhibitor, with approximately 25% of the fertilized eggs being affected. As the concentration of the inhibitor was increased, a higher percentage of the cells in the preparation showed visible abnormalities. The findings with regard to the appearance of the FE, in about 5-15 minutes following fertilization, are illustrated in Fig. 1, where Panel A presents the controls without (Left) and with $Me_2SO$ (0.1%; Right) contained in the medium. Panel B shows typical microscopic fields of the eggs fertilized with 0.001 mM (Left) or 0.05 mM (Right) of the inhibitor in the sea water. The most striking abnormality

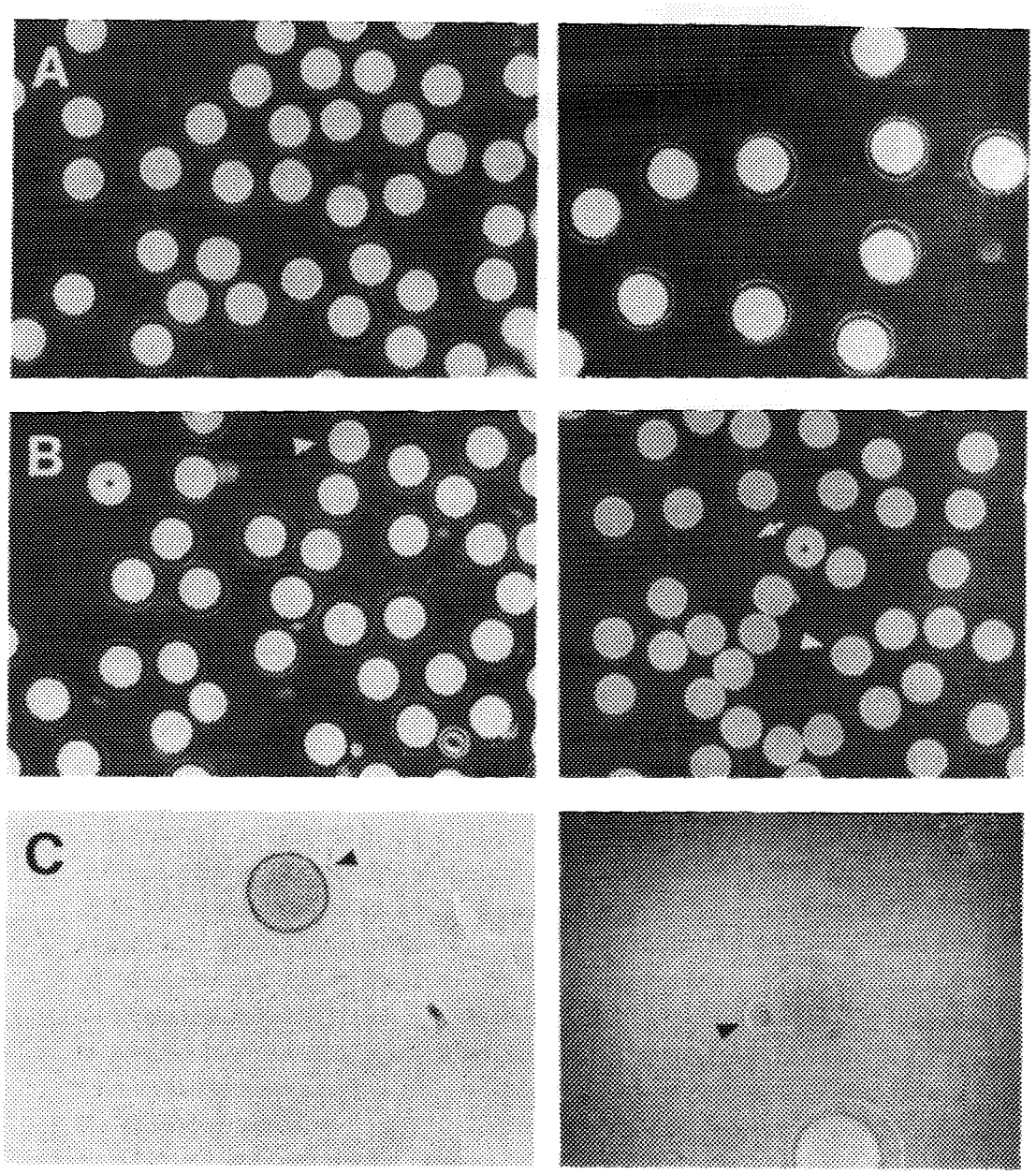


Fig. 1. Fertilization envelope abnormalities in *S. granularis* eggs fertilized in the presence of low concentrations of an active-site-directed, transglutaminase inhibitor: (1,3,4,5-tetramethyl-2-[(2-oxopropyl)thioimidazolium] chloride. Panel A: Control eggs after 5 min of fertilization in sea-water (left; x 80 magnification) or with 0.1% $Me_2SO$ in sea water (right; x 100). Panel B: when 0.001 mM (left; x 80 ) or 0.05 mM inhibitor (right; x 80 ) in 0.1% $Me_2SO$ was added to the water, explicit wrinkling of FE (examples are marked by triangular arrowheads) was evident by 5 min. In addition, fragility of FE could be inferred from finding significant numbers of denuded eggs (examples are marked by black asterisk) and empty FE's (marked by white arrow in the righthand panel B). Panel C: Illustrates the irregular appearance of FE in the presence of 0.001 mM of the inhibitor (left; x 150) and the partial extrusion of the eggs (right; x 150) at 0.05 mM of the inhibitor at 15 min after fertilization.

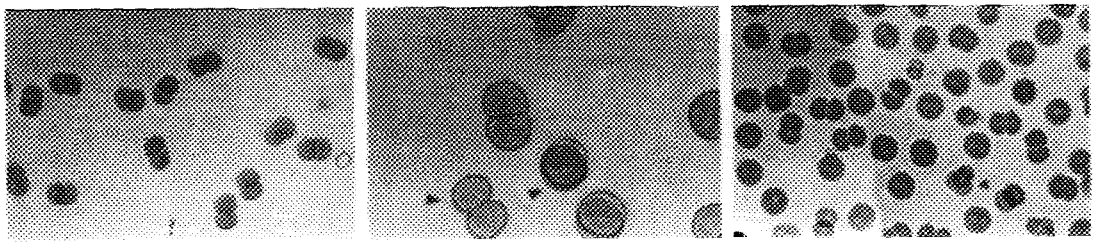

Fig. 2. Irregularities in cell division were evident in the presence of the transglutaminase inhibitor by about 45 min after fertilization. Middle Panel: 0.001 mM inhibitor (x 130 magnification; wrinkling of FE's are marked by black triangular arrowheads). Righthand Panel: 0.05 mM inhibitor (x 60). Left Panel: Control cells (with 0.1% $Me_2SO$ solvent in the sea water; x 65 ).

is a marked wrinkling of the FE, examples of which are indicated by arrow-heads in the photographs. It may be presumed that the folded appearance of the FE is due to a failure of this extracellular protective layer to become hardened by undergoing I-T transition (8, 9, 12). The fact that there are many denuded cells (an example of which is marked by asterisk) as well as empty FE's (indicated by the arrow) in the preparation would seem to be in line with the interpretation that the FE's became abnormally fragile. Panel C illustrates further the irregular appearance of the FE's (Left; at 0.001 mM inhibitor) and the extrusion of the fertilized egg (Right; at 0.05 mM inhibitor) through the envelope.

Cell division by the 2-cell stage was also impaired in the presence of the inhibitor (Fig. 2, 0.001 mM L-682777 in the middle, 0.05 mM on right). The most notable feature was the high percentage of multinucleate cells. With 0.05 mM of the inhibitor, approximately half of all cells became polynucleated. Similar results were obtained (carried out earlier on *A. punctulata* eggs; data not shown) with 0.1 mM of another, albeit less effective, inhibitor of transglutaminase thought to be directed against the active center of the enzyme: 2-[3-(diallylamino)propionyl]benzothiophene (4, 13) and also with the competitive substrate of transglutaminase-catalyzed protein cross-linking reactions: dansylcadaverine (or N-(5-aminopentyl)-5-(dimethylamino)-1-naphthalensulfonamide) at 2 mM concentration in the medium. The finding that these different types of inhibitors of transglutaminase produced the disparate abnormalities of FE wrinkling as well as the derangment of normal cell division, may be taken to suggest that different forms of transglutaminases might function in protein cross-linking reactions at various stages during the program of embryonic development.

## References

1. Campbell-Wilkes, L. K. (1973) Ph. D. Dissertation, Northwestern University, Evanston,IL (Xerox University Microfilms No 73-30, 763).
2. Lorand, L., and Stenberg, P. (1976) Handb. Biochem. Mol. Biol, 3rd Ed. 2, 669-685.
3. Takeuchi, K., Cariello, L., and Lorand, L. (1982) Fed. Proc., Fed. Am. Soc. Exp. Biol. 41, 1453 (Abstract 6913).

4. Cariello, L., Wilson, J., and Lorand, L. (1984) Biochemistry 23, 6843-6850.
5. Cariello, L., Velasco, P. T., Wilson, J., Parameswaran, K. N., Karush, F., and Lorand, L. (1990) Biochemistry 29, 5103-5108.
6. Lallier, R. A. (1970) Exp. Cell Res. 63, 460-462.
7. Lallier, R. A. (1971) Experientia 27, 1323-1324.
8. Battaglia, D. E., and Shapiro, B. M. (1988) J. Cell.Biol. 107, 2447-2454.
9. Mozingo, N. M., and Chandler, D. E. (1991) Dev. Biol. 146, 148-157.
10. Barsigian, C., Stern, A. M., and Martinez, J. (1991) J. Biol. Chem. 266, 22501-22509.
11. Freund, K. F., Doshi, K. P., Gaul, S. L., Claremon, D. A., Remy, D. C., Baldwin, J. J., Pitznberg, S. M., and Stern, A. M. (1994) Biochemistry 33, 10109-10119.
12. Vernon, M., Foerder, C., Eddy, E. M., and Shapiro, B. M. (1977) Cell 10,321-326.
13. Lorand, L., Barnes, N., Bruner-Lorand, J.A., Hawkins, M., and Michalska, M. (1987) Biochemistry 26, 308-313.