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Using hemocytes of the marine bivalve mollusc Mytilus californianus, cell aggregation (clump formation) and cell adhesion to the substratum have been exploited as indicators of hemocyte activation. Two intracellular signal transduction pathways, the cAMP pathway and the phospholipid/Ca^{++} pathway, have been found to be involved in hemocyte activation. During activation intracellular levels of cAMP increase. Based on the synergistic and competitive effects of caffeine and adenosine or adenosine analogues on cell behavior, it is inferred that caffeine inhibits cell activation as a result of its binding to adenosine receptors. The putative adenosine receptor may initiate a cascade of reactions to activate adenylate cyclase and generate cAMP. The phospholipid/Ca^{++} pathway was made evident by the activation ability of two protein kinase C activators, PMA and A23187. Both drugs overcame the inhibitory effects of caffeine on hemocyte activation. Extracellular calcium ions may also be critical to hemocyte activation, since the calcium channel blocker, verapamil, influenced

hemocyte aggregation and adhesion, and 50 mM calcium could induce hemocyte adhesion in 10 mM caffeine. Cell surface carbohydrates also play a role in hemocyte adhesion and aggregation. These two cell behaviors have been found to be inhibited by $NaIO_4$ and N-acetyl-glucosaminidase, and promoted by neuraminidase and hyaluronidase. In addition, cytoskeleton assembly is also involved in these two cell activities.

Although some evidence implies indirectly that hemocyte aggregation and adhesion are distinct behaviors, the adhesion molecules putatively involved remain to be identified, so cannot be relied upon to distinguish these two cell behaviors. However, these two cell events likely utilize the same or very similar activation pathways. Not only do both behaviors always occur simultaneously, but their inhibition and enhancement were effected by the same reagents in most cases. In addition, as demonstrated in sponge cell aggregation or myoblast aggregation, Mytilus hemocyte aggregation appears to be a two-step process that advances through weak aggregation to cohesive aggregation, and which is modulated by temperature and dependent on divalent cations and metabolic energy.

THE CELL ACTIVATION MODEL OF HEMOCYTE AGGREGATION AND ADHESION IN THE CALIFORNIA MUSSEL, *Mytilus californianus*.

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THE CELL ACTIVATION MODEL OF HEMOCYTE AGGREGATION AND ADHESION IN THE CALIFORNIA MUSSEL, *MYTILUS CALIFORNIANUS*.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Recently, in order to understand the basis of the complexities of immune regulation in vertebrates, the evolutionary origins of immune responses have been intensively investigated in invertebrates (Ratcliffe et al., 1985; Solomon, 1986; Reinisch & Litman, 1989). Although immunoglobulin has not been detected in invertebrates, responses including graft rejection and inflammatory reactions indicate that invertebrates possess primitive immune mechanisms (Bayne, 1983; Ratcliffe et al., 1985). Comparisons of the immune systems of invertebrates and vertebrates reveal major morphological differences in their blood cells. With less effective humoral factors in invertebrates, cellular immunity appears to play a more important role than it does in vertebrates.

Most bivalves and gastropods have shells to protect them from mechanical damage and shield them from environmental stress. In addition, they possess mucus glands and ciliated epithelia on their exposed surfaces which secrete and transport sticky mucus when the animal is irritated. However, shells and such specialized surfaces cannot always prevent pathogen invasion. When external barriers are breached, internal defense systems are needed to reduce contamination and to handle biotic or abiotic materials which gain entry to

the body. These internal defense systems utilize hemocytes (blood cells) and humoral mechanisms to eliminate invading particles (Sminia & van der Knaap, 1987). Such reactions resemble some elements of natural immunity in vertebrates. Bivalves and gastropods have open circulatory systems in which their hemolymph (blood and body fluid) passes from the arteries into large sinusoids thus bathing major organ systems.

Characterization of bivalve and gastropod hemocytes

In addition to providing cellular defenses (Bayne, 1983; Cheng, 1984; Sparks & Morado, 1988), the hemocytes serve a variety of roles including digestion and nutrient transport (Cheng & Cali, 1974; Bayne, 1983), excretion (Narain, 1973), as well as shell repair (Watanabe, 1983). It is to be expected that several different cell populations might coexist and perform these physiological functions in a cooperative manner. For many species of molluscs, little is known about the origin of the hemocytes (Sminia, 1981), or their ontogeny (Mix, 1976). With respect to bivalves, this question remains unresolved: are these cells newly formed at some hemopoietic center(s) or are they products of divisions of cells in circulation (Cheng, 1981)? Environmental conditions are known to influence the number of molluscan hemocytes in circulation. For example, exposure to higher temperatures rapidly increases hemocyte numbers (Davies & Patridge, 1972). Leucocytosis can be induced also by wounding, parasitism (Renwrantz & Cheng, 1977; Stumpf & Gilbertson, 1980) and other abiotic factors (Wolmarans & Yssel, 1988).

The hematology of more than 30 species of molluscs has been studied (Cheng, 1981; Sminia, 1981; Rasmussen et al., 1985; Auffret, 1988). However, no single taxonomic system has been generally accepted for molluscan hemocyte classification. In the absence of clear definitions for the several types of molluscan hemocytes, various morphological features have been

used by different investigators to designate cell types. Thus, there is still no agreement on how many types of hemocytes can be distinguished in molluscs.

Even though problems of hemocyte nomenclature have not yet been solved, two main schemes are broadly followed for molluscan hemocyte classification. The first was contributed by Cuénot (1891; cited by Narain, 1973). He characterized three types of molluscan hemocytes, namely finely granular, coarsely granular and lymphocyte-like hemocytes. The second scheme simply separates molluscan hemocytes into granulocytes and hyalinocytes (Takatsuki, 1934; cited by Narain, 1973). These cell types are commonly distinguished on the basis of morphological, behavioral, or histochemical differences. Yet such features may vary between closely related species. For example, the diameters of granulocytes vary from 9μm to 70μm possibly reflecting stages of maturation. But such characters are also influenced by the methods used to prepare cells for observation (Cheng & Auld, 1977). Cytoplasmic granules are also variable. Granulocytes may have two or three combinations of acidophilic, basophilic and neutrophilic granules (Foley & Cheng, 1974; Bayne et al., 1979). It has been suggested that all of the granules go through an alkaline phase prior to becoming acidic (Yoshino & Cheng, 1976), but fixatives may also affect the binding affinity of dyes with granules (Foley & Cheng, 1972).

Any comprehensive system for the classification of molluscan hemocytes must draw on more than morphological and behavioral criteria. Data obtained from ultrastructure studies (Cheng & Foley, 1975), cell surface marker labeling (Yoshino & Granath, 1983), specific gravity (Cheng et al., 1980), as well as intracellular acid phosphatase and lysozyme levels (Cheng & Downs, 1988) collectively provide a basis for the classification of hemocyte subpopulations. It is now clear that hemocytes may be quite distinctive even between closely related molluscan species (Auffret, 1988).

In the absence of a widely accepted classification, molluscan hemocytes are generally allocated into two broad types, granulocytes and hyalinocytes (Table I-1).

Cellular immunity in bivalves and gastropods

Protective cellular responses in molluscs include inflammation (Cheng, 1983; Sparks & Morado, 1988), wound repair (Sparks, 1972; Bayne, 1983), phagocytosis (Fisher, 1986; Bayne, 1990), encapsulation (Sminia, 1981; Feng, 1988), and cell clumping (Bang, 1961; Narain, 1973).

Inflammation and wound repair

Generally, the inflammatory process in invertebrates is similar to that of vertebrates. Leukocytosis, the increase in numbers of leukocytes in circulation, is one of the initial responses of inflammation. When molluscan tissue is injured, with or without microbial infection, the initial step of wound healing is local muscular contraction (Sminia, 1981). Hemocytes then accumulate near the lesion and infiltrate the wound site. These early events may be accompanied by edema (Sparks & Morado, 1988). The aggregated hemocytes clump together to form a plug at the site of the wound, closing it and preventing further hemorrhage and contamination (Sparks, 1972). Such hemostatic mechanisms are important in both vertebrates and invertebrates, since loss of much blood or hemolymph would retard or preclude recovery from injury. Hemocyte clots in invertebrate are quite different from vertebrate platelet clots. First, no tight junctions are formed in hemocyte plugs (Loker et al, 1982). Second, no coagulation proteins such as fibrinogen and thrombin are involved (Narain, 1973). Third, the aggregation of hemocytes is reversible. Most aggregated cells later disperse as wound repair progresses (Ruddell, 1971; Feng & Feng, 1974).

During the late phase of wound healing, molluscan hemocytes at the margin of a plug become flattened. They

phagocytose cell debris, injured tissue, or invasive microorganisms (Sparks, 1972). Oyster cells can release copper in the lesion (Ruddell, 1971), yet the function of this copper is not known. The hemocyte plug remains in the wound area(s) until adjacent epithelial cells divide and migrate over the wound (Bayne et al., 1979; Fisher, 1986). Some of the hemocytes that remain at the wound area(s) differentiate into fibroblast-like cells, depositing collagen fibrils to form collagenous connective tissue (Sminia, 1981; Fisher, 1986).

Phagocytosis

The phagocytic ability of molluscan hemocytes was described early by Haeckel (1862, cited by Cheng, 1984). Stauber (1950, cited by Bayne, 1983) injected India ink into Crassostrea virginica to trace the fate of ink particles, and found that hemocytes subsequently accumulated to phagocytose the ink particles. It is now recognized that phagocytosis plays an important role in molluscan internal defense (Bayne, 1990). Three uptake mechanisms have been suggested to occur in phagocytosis by molluscan cells. The first was described by Bang (1961). Some bacteria, though not all, initially adhere to the surface of hemocytes of C. virginica, then glide along filopodia and are taken into the cytoplasm to form phagosomes. The second mechanism (Cheng, 1975) is similar to Bang's observation, but no filopodia are involved. According to the third model, granulocytes and hyalinocytes may form funnel-like pseudopods through which red blood cells can be taken into the cytoplasm (Schoenberg & Cheng, 1980). While all of the hemocytes of C. virginica, C. gigas and Mercenaria mercenaria are phagocytic, granulocytes seem to be the most important phagocytes (Foley & Cheng, 1975; Sminia, 1981).

Phagocytosis is regarded as the first line of internal defense in vertebrates and invertebrates. However, not all invasive particles or organisms are phagocytosed. Some parasites can successfully avoid immunological attacks by

their host, possibly by expressing host-like antigens on their surfaces. Such molecular mimicry may be developed during co-evolution of the host and parasite (Bloom, 1979; Yoshino & Bayne, 1983). Some parasites can release immuno-suppressive factors to inhibit the immune responses of their hosts, and some parasites can acquire antigen(s) from their hosts (Yoshino, 1984; Bayne et al., 1986).

Phagocytosis by molluscan hemocytes occurs in four sequential stages: attraction or random collision, adherence (non-self recognition), endocytosis and intracellular digestion.

Although chemotaxis to bacterial products has been reported for some invertebrate phagocytes (Schmid, 1975; Cheng & Howland, 1979), the putative chemotactic factor(s) have not been isolated. Some attractants may come from bacterial cell walls and envelopes (Cheng & Howland, 1982). When the hemocytes of C. virginica were treated with cytochalasin B and colchicine, chemotactic responses were inhibited. It was inferred that chemotaxis was dependent on cytoskeleton assembly, a major process in cell movement (Cheng & Howland, 1982).

Selective adherence between bacteria and hemocytes of C. virginica was first described by Bang (1961). Carbohydrate-binding proteins (lectins) occur on molluscan hemocytes (Sminia, 1981; Vasta et al., 1982; Cheng, 1983), and can be released from hemocytes (Leippe & Renwrantz, 1988; Mitra & Sarkar, 1988). Such molecules may serve as cell surface receptors or as humoral components which bind to foreign particles (Bayne et al., 1984) to enhance phagocytosis. In fact, plasma factors can bind with foreign particles to influence the outcome of an encounter with hemocytes in vitro and in vivo (Granath & Yoshino, 1984; Bayne et al., 1986). For example, opsonins have been demonstrated in the plasma of C. gigas (Hardy et al, 1977), L. stagnalis (Sminia et al., 1979), Helix pomatia (Renwrantz et al., 1981), and B. glabrata (Fryer

& Bayne, 1989). Also, some agglutinins have been suspected to have opsonic activity (Pauley et al., 1971; Schmid, 1975). Therefore the agglutinin and the opsonin may be the same molecule (Bayne, 1981). Interestingly, phagocytosis by molluscan hemocytes can occur, at least in some cases, in the absence of plasma factor(s) (Abdul-Salam & Michelson, 1980; Boswell & Bayne, 1984; Fryer & Bayne, 1989).

Encapsulation

When non-self particles invade molluscs or are transplanted experimentally, phagocytosis or encapsulation is normally induced. If particles are too large to be phagocytosed, they may be gradually surrounded by hemocytes which spread to cover a large surface area on the particle. The latter is eventually immobilized and encapsulized by a multi-layered cell sheet. The rate and extent of encapsulation is dependent on the compatibility of the foreign particle and the host, and on the immunological capacities of the host. For example, parasite encapsulation occurs more rapidly and is more extensive in resistant strains of snail than in susceptible strains (Bayne et al., 1980; Loker et al., 1982). Before electron microscopy was used for studies of encapsulation, it was thought that more than one type of hemocyte is involved in capsule formation (Cheng & Rifkin, 1970). Now, granulocytes (amoebocytes), and not hyalinocytes, are recognized to be the dominant cell type involved in this process (Cheng & Garrabrant, 1977).

Encapsulation is a complex cellular interaction. It is composed of several steps including non-self recognition (cell-particle), and self recognition (cell-cell), and is accompanied by extracellular and intracellular digestion. However, the details of encapsulation in molluscs are still little known. It has been reported that amoebocytes, fibroblasts, and myofibroblasts appear in the capsule, and the intercellular substances are synthesized by amoebocytes which

are transformed into fibroblasts (Sminia et al., 1974). In contrast, Harris (1975), who studied B. glabrata / Angiostrongylus cantonensis interactions, reported that neither extracellular connective tissues nor myofibroblasts are found in these capsules. Regardless of the type of encapsulation, hemocytes involved in the capsule interdigitate with each other, but no tight junctions or desmosomes are formed (Bayne, 1983). Locally secreted mucopolysaccharides and reticular fibrous materials facilitate immobilization (Cheng & Rifkin, 1970).

Hemocyte clump formation

The first report of molluscan cell clumping was published by Geddes (1880, cited by Narain, 1973). Geddes described cell clumps as "plasmodia" because he observed hemocytes partially or entirely fused together to form clots. In fact, clotted hemocytes only attached to each other by their hyaline filopodia (Michel, 1888, cited by Cheng, 1984). Further studies revealed that after hemocytes establish contact, they are drawn together by the contraction of filopodia (Drew, 1910, cited by Cheng, 1984). Small clumps of hemocytes then aggregate to form larger masses (Dundee, 1953).

In molluscs, cell clump formation occurs in vivo in response to microbial injection (Bang, 1961). Such aggregation is also induced in vitro (Narain, 1973; Seifert, 1983). Actually, cell clumping is morphologically similar to encapsulation and cell clotting in wound repair. As soon as hemolymph is harvested from a mollusc, hemocytes rapidly aggregate. The initial inducer is still not clear. Within the clumps, cells are tightly cohesive and can be dispersed only with difficulty by pipetting or shaking (Vasta et al., 1984; Leippe & Renwrantz, 1988). However, the cellular associations are reversible both in vivo (Bang, 1961) and in vitro (Narain, 1973). After hemocyte clumps attach to solid surfaces, some cells migrate out of the clumps within minutes. Although

hemocyte clumping has long been known in molluscs, attempts to unravel its biochemical basis are few (Seifert, 1983).

Cellular immunity in other invertebrates

Besides molluscs, the immunity of arthropods, annelids, echinoderms, and some smaller taxa have been studied. From those studies, it is clear that these animals have similar cellular defenses including phagocytosis, cell clotting and/or nodule formation, encapsulation, and cell-mediated cytotoxicity. Their blood cell types vary in morphology, cell behavior, and maybe with respect to function (Smith, 1981; Ratcliffe et al., 1985; Johansson and Söderhäll, 1989b).

A. Crustaceans and insects

The mechanisms of cellular defenses in arthropods are similar to those in other mentioned invertebrates except for the prophenoloxidase (proPO) system (Johansson and Söderhäll, 1989a). Cellular defense reactions in arthropods are often accompanied by melanization. The melanin is synthesized by phenoloxidase (PO) that can be activated from its pro-enzyme, proPO, by microbial cell wall components such as bacterial lipopolysaccharides, and peptidoglycans, also fungal β-1,3-glucans (Söderhäll and Smith, 1986). The function of the proPO system may be to directly mediate arthropod cellular immunity (Söderhäll and Smith, 1986; Lackie, 1988). Encapsulation promoting factor (EPF) has been partially purified from some arthropods. EPF causes hemocytes to adhere to foreign surfaces and may induce them to flatten to form capsules (Ratner and Vinson, 1983).

a. Insects

Nodule formation as a clearance mechanism has been well studied in insects (Ratcliffe et al., 1985). In Galleria mellonella this is a two-stage process. When granular cells randomly contact invading bacteria, they discharge some

granular substance and become sticky, forming a clot that can entrap bacteria (Ratcliffe & Walters, 1983). The initial stage of nodule formation is completed extremely rapidly, unlike chemotaxis. The second stage is similar to an encapsulation response, phagocytic cells engulfing and digesting entrapped bacteria (Walters & Ratcliffe, 1983). Several antibacterial molecules, cecropin, attacin, and diptericin are produced by insect hemocytes. All of them are broad-spectrum antibacterial materials (Trenczek, 1986)

b. Crustaceans

Cells contributing to immune responses include hyaline (H) cells, semigranular (SG) cells, and granular (G) cells (Amirante, 1986). In the crab Carcinus maenas, invading bacteria are localized in hemocyte (H cells and SG cells) nodules, which are formed in the gills and in the sinuses between the hepatopancreatic tubules, and then are digested. This process is dependent on the presence of plasma opsonin, calcium ions, and pH (Amirante, 1986). It has been proposed that the SG cells are the first proPO secreting cells. When SG cells contact foreign particle(s), the proPO is secreted into the plasma and is converted to PO. The active PO then amplifies the release of proPO by degranulation of S and SG cells (Johansson and Söderhäll, 1989b). Crustacean hemocytes also can aggregate in vitro when they are exposed to wound factor(s) during bleeding. So nodule formation and function in crabs resemble these processes in insects (Ratcliffe et al., 1985).

B. Echinoderms

The coelomocytes of echinoderms will aggregate in vivo after injury or in vitro after removal from the coelomic cavity. Coelomocyte clumping is associated with hemostasis and injury repair in sea cucumbers (Noble, 1970) and in sea stars (Kanungo, 1982). Bladder shaped coelomocytes (amoebocytes)

transform to filiform cells and form calcium-independent disulfide linkages between cells (Boolootian & Giese, 1959). However, later experiments revealed that EDTA can partially inhibit cell clumping (Noble, 1970; Kanungo, 1982). So far, there are few biochemical data on such cell-cell interactions.

Cells dissociated, enzymatically or non-enzymatically, from the blastulae of echinoderms can reaggregate and then reconstitute a normal embryo (Turner, 1987). Such cell reaggregation is a two-step process. As in sponge cell reaggregation, Ca^{++} is not required in the first step, and the second step is Ca^{++}-dependent. Freshly dissociated cells are round and without microvilli. Within 90 min, aggregated cells become flattened and reform microvilli. If such cells are treated with azide or fixed, they remain spherical, and reaggregation is blocked (Watanabe et al., 1982).

Cell-cell adhesion and cell-substratum adhesion

One component of cellular immunity is cell-cell recognition which includes cell-cell contact and cell-cell adhesion. These cell behaviors regulate the function of the vertebrate immune system. For example, in the process leading to antigen presentation, an antigenic particle is first phagocytosed by an antigen-presenting cell. After digestion, epitopes are presented on the cell surface. When helper T-cells recognize antigens, they contact B cells, and produce stimulatory interleukines which lead B cells produce specific antibodies against the antigen.

The importance of cell adhesion is well known in evolutionary biology and developmental biology. According to accepted dogma, the metazoa arose by the aggregation of single cells whose association was mediated by specific intercellular adhesion molecules (reviewed by Edelman, 1983). This cell behavior is critical in development and differentiation also, because an isolated somatic cell cannot differentiate. It has to encounter other cells either homotypic or heterotypic. Of

course, attachment to a proper substratum, such as an extracellular matrix, is also important for specific cell differentiation.

As mentioned above, two categories of cell adhesion can be recognized, namely cell-cell adhesion, and cell-substratum adhesion. It appears that most cell-cell adhesions are mediated by cell adhesion molecules (CAMs), whereas integrins and substratum adhesion molecules (SAMs) are mainly associated with cell-substratum adhesions. Based on accumulated evidence, CAMs and SAMs belong to separate families of molecules (reviewed by Edelmam, 1983). An increasing number of CAMs have been isolated. Neural cell adhesion molecule (N-CAM) is one which has been well characterized. Like other CAMs, N-CAM is a cell surface glycoprotein of relatively high molecular weight. N-CAM binding is homophilic, and Ca^{++}-independent (Hoffman & Edelman, 1983; Rutishausen et al., 1988). However, calcium is necessary for liver cell adhesion molecules (L-CAMs). SAMs include glycosaminoglycans (GAGs), fibronectin, laminin, and collagen. Although some of them are detectable on cell surfaces, most are major components of the substratum in connective tissues.

Cell-cell adhesion has been ascribed to a variety of biological surface factors including surface charge (Bell, 1983; Greig & Jones, 1977), divalent cation concentration (Shozawa & Suto, 1990), extracellular carbohydrate (Brandley & Schnaar, 1986; Rutishausen et al., 1989), and adhesion molecules, as well as some cytosolic factors including calcium and cAMP (Dunham et al., 1983). Theoretically, cell-cell adhesion may utilize either non-specific or specific adhesion mechanisms. Non-specific adhesion such as certain colloidal particles adhesion is influenced by electrostatic repulsion, van der Waals forces, and steric stabilization between hydrophobic surfaces (Bell, 1983). However, since the surface charge of most cells is negative, these forces actually prevent cells from coming close together. Specific adhesion

results from specific surface receptor and ligand interactions between cells. The binding is strong enough to overcome non-specific repulsion. When cells make contact by collision, or centrifugation, the distance between two adhesion molecules on different cells is close enough to allow binding. In the other case, when the cells are activated by signal molecules, like hormones or antigens, cells probably alter the gradient of ions inside the cells and on cell surfaces, thus reducing the repulsive force and promoting cell contact.

Cell-cell adhesion (cell aggregation) in various cell types

Cell aggregation occurs not only with invertebrate blood cells including hemocytes, amoebocytes, and coelomocytes, but with a great variety of others. Among the most extensively studied are the slime molds, sponge cells, ovotestis cells of Helix pomatia, and mammalian blood platelets.

A. Slime molds

The life cycles of slime molds include distinct reproductive (colonial) and vegetative (solitary) forms. As long as their food sources (bacteria) are abundant, the amoebae (vegetative form) live as single cells, growing and dividing. During starvation, amoebae secrete cAMP, which chemotactically attracts nearby amoebic cells (Loomis, 1979). During the processes of migration, the accumulating cells secrete more cAMP. The pulse of cAMP causes more cells to move toward the central area of higher cAMP concentration. The aggregated unicellular amoebae fuse thus transforming non-social vegetative forms to the social multicellular (syncytial) organism. Cells in specific positions then differentiate into a fruiting body containing stalk or spore cells. At this phase of their life cycle, they can survive periods of starvation or other stressful environmental conditions.

During the early stage of development, amoebae of Dictyostelium discoideum express two types of cell-cell adhesion molecules on their surfaces. One is divalent cation-dependent, and the other is divalent cation-independent. The latter is expressed during cell aggregation, and has been characterized as a surface glycoprotein of M.W. 80,000. It regulates cell-cell adhesion via homophilic binding. An octapeptide whose amino acid sequence is YKLNVNDS has been revealed to be the cell-cell binding domain. The other EDTA-restricted cell-cell adhesion molecule is gP150. These molecules accumulate primarily at the contact site between cells. Because specific anti-gp150 Fab cannot completely block cell aggregation, it is considered to mediate cell cohesion at the post aggregation stage (reviewed by Siu, 1990).

B. Sponge cells

Marine sponges were the first to be used for in vitro studies of cell aggregation, and cell recognition (Wilson, 1907, cited by Brandley, 1986). Sponge cells dissociated by either mechanical forces or proteolytic enzyme treatment can spontaneously reaggregate to form intact organisms. Initial contacts result from random collisions (Müller, 1980). When dissociated cells from two species are mixed, species-specific reconstitution is the result of a sorting out process. This step is considered to be the second stage of cellular recognition in sponge reaggregation (reviewed by Müller, 1980).

There are two sequential processes in sponge cell aggregation, namely primary aggregation, and secondary aggregation. The former is the initial stage which leads to small aggregate formation. It requires divalent cations like calcium, strontium, or barium, but no soluble organic factors appear to be involved (Takeichi, 1977). It is a pH-sensitive reaction. The optimal pH is around 8.0. If the pH value is below 6.5 or higher than 9.0, primary aggregation is

inhibited. Although pre-incubation with proteases or carbohydrate-hydrolyzing enzymes failed to block primary aggregation, antiserum against a 17,000 MW surface molecule of the sponge, Geodia cydonium, could strongly inhibit the formation of primary aggregates.

From sponges, two types of molecules, aggregation factors (AF) and aggregation receptors (AR) have been isolated which are involved in secondary aggregation. The aggregation factors are soluble glycoproteins having two subunits (MW 65,000, and 60,000) linked by a disulfide bond. The homologous binding of the aggregation factors is Ca^{++}-independent (Takeichi et al, 1979). The aggregation receptors are membrane-bound glycoproteins. Binding studies of aggregation factors and aggregation receptors reveal a Ca^{++}-dependent reaction. Recent studies have revealed that AF-AR interactions in sponges are regulated by protein kinase C (Gramzow et al., 1988). When the L-lysine and L-arginine at the N-terminal of the aggregation factor are removed by specific protease, the activity of the aggregation factor is lost. The D-glucuronic acid of the aggregation receptor is also important in such binding.

C. Molluscs

Since the common methods for dissociating vertebrate and invertebrate tissues were found to be unsuitable for snail tissues, a modified dissociation medium was formulated by Farris (1968). She found that substitution of sodium gluconate for NaCl in the physiological salt solution improved the dissociation of snail tissues. When she removed the modified dissociation medium by centrifugation, the cells clumped together. Tissue sections of ovotestis and of cell-clumps revealed that the same cells were present in both, and the histological structures were similar. The cells could recognize each other and move to their correct position relative to each other during reorganization.

D. Platelets

Platelet aggregation is a major event in blood clot formation in vertebrates. This aggregation has been well investigated and has been found to include complicated cascade reactions. As platelets are exposed to high concentration of ADP, their net negative surface charges reduce and their shapes change (Gaarder et al., 1961; Seaman, 1967). During aggregation, fibrinogen and calcium ions are required (Seaman and Brooks, 1970; Kinlough-Rathbone et al., 1977). Fibrinogen can crosslink platelets by binding to membrane receptors. Also fibrinogen is converted to fibrin by thrombin which is a proteolytic enzyme in plasma. Platelets serve to contract the clot by activating their actin and myosin to pull fibrin strands together, and also by compressing the fibrin reticulum into a compact mass. In addition, the entrapped platelets secrete fibrin-stabilizing factor to strengthen the fibrin meshwork structure.

E. Myoblasts

The fusion of myocytes to form myotubules is a main process in the development of skeletal muscles. Like slime molds, cultured myoblasts fuse together, and then differentiate when certain essential nutrients are depleted in their growth media. The differentiation of myoblasts can be inhibited by thiodigalactoside which does not affect myoblast proliferation. The aggregation of chick myoblasts is mediated by dual adhesion systems. One is Ca^{++}dependent, and the other is Ca^{++}-independent (Gibralter & Turner, 1985). There are two types of molecules (CAMs) identified from chick myoblasts whose adhesion activities can be interfered with by specific antibodies (Pizzey et al., 1988).

Cell recognition

The involvement of extracellular domains of integral membrane proteins in cell recognition and in cell adhesion has

been unravelled in the last decade. One example is the specific binding of intergrins to an R-G-D containing protein like fibronectin (Hynes, 1987; Kishimoto et al., 1989). Three families of cell surface molecules including the immunoglobulin family, intergrin family, and selectin family have been recently identified on T cell and other immune cells as immune response regulators (reviewed by Springer, 1990). Due to the complex structure of proteins caused by variations in amino acid sequence, proteins were considered to be the only molecules able to deal with such specific interactions. Although most cell surface proteins are glycoproteins, the possible role of carbohydrates in cell-cell interactions was neglected. Most cell membranes contain glycoproteins and glycolipids which display carbohydrate moieties located on their extracellular terminals to form a carbohydrate outer layer (glycocalyx). Although the basis of molecular complexity in carbohydrates differs from that of proteins, carbohydrates are also involved in cell-cell interactions. Actually, the structure of carbohydrate moieties on glycoproteins or glycolipids is quite complex due to their special branched structure. Also, the addition of sulfates, phosphates and acetyl groups to the carbohydrate moiety increases their structural complexity (reviewed by Brandly & Schnaar, 1986). On the other hand, the peripheral layer of carbohydrates may play the opposite role in cell-cell interactions, since the location and thickness of the glycocalyx may prevent protein interactions between cells (Rutishauser et al., 1988). In addition, sialic acid, a major carbohydrate component on cell membranes, generates negative charges on cell surfaces causing repulsion between cells (Greig & jones, 1977; Bell, 1983).

Other evidence of carbohydrate involvement in cell interactions is obtained from studies of the biological significance of animal, plant, and microbial lectins. Lectins are a group of proteins that interact with glycoproteins, glycolipids or pure carbohydrates by binding to specific

carbohydrate residue(s). Most lectins recognize terminal nonreducing saccharides, though some have extended binding sites which can bind with the subterminal sugars. A few lectins recognize carbohydrate sequences near the peptide moiety of a glycoprotein (Lis & Sharon, 1986). Therefore, molecular shape, not just sequence, is also important for lectin-carbohydrate interactions. However, it appears that the specificities of animal lectins are limited to a few sugars. For example, lectins which recognize mannose 6-phosphate, mannose, L-fucose or N-acetylgalactosamine are widely present in mammalian cells (Simpson et al., 1978). B-galactoside-binding lectins isolated from vertebrates share a common oligomeric structure leading to immunological cross-reactivity (Hirabayashi et al., 1987). Also, many invertebrate lectins share a common specificity for sialic acid (Yeaton, 1981; Miller, 1982; Ravindranath et al., 1985).

Known lectin involvement in cell-cell interactions includes slime mold differentiation (Rosen et al., 1976; Barondes et al., 1978), sponge cell reaggregation (Bretting et al., 1976; Varner et al., 1988), human blood clotting (Gartner et al., 1981; Jaffe et al., 1982), lymphocyte recirculation (Ashwell & Harford, 1982; Streeter et al., 1988), species-specific binding of sperm to egg (Vacquier & Moy, 1977; Rosati & de Santis, 1980), pathogen-host adhesion (Jungery et al, 1983; Villata & Kierszenbaum, 1983; Ward et al., 1987), virus infection (Varghese et al., 1983; Mitsuya et al., 1988), and bacterial adhesion (Eshdat et al., 1978).

Cell activation

The term cell activation has been used to describe a variety of changes to the resting state of a cell. Appropriate stimulation may cause morphological changes (Shaw et al., 1990), functional changes (Gardner, 1989), or may induce new cell activities (Kaibuchi et al., 1981; Morrison et al., 1989). In molluscan hemocyte aggregation and adhesion,

hemocytes change from spherical to flattened, and their behavior is altered. Therefore, morphological, functional and behaviour changes of mussel hemocytes can be regarded as indicative of one kind of cell activation.

Thesis goal and hypothesis

Both hemocyte aggregation and adhesion to foreign surfaces *in vitro* are well described in invertebrates. However, the mechanisms by which naive hemocytes are activated are not clear. In the marine mussel, *Mytilus californianus*, hemocytes aggregate and/or adhere to foreign surfaces within seconds of removal from the animal. The aggregated cells can later migrate out of aggregates and adhere, but the adherent cells generally do not move together to form aggregates. Obviously, aggregation and adhesion are two different cell behaviors. Hemocyte aggregation is a form of cell-cell adhesion which depends on self-recognition. Cell adhesion, an interaction between cell and substratum, involves non-self recognition. Thus, the first question addressed in this thesis is: "What is the difference between cell aggregation and cell adhesion in *Mytilus*?"

Both cell aggregation and adhesion seem to occur spontaneously. However, they are never observed *in vivo* unless the animal is wounded. Therefore, the concept of a "wound factor" has been accepted, though the character of this putative inducer has not yet been realized. The second question to be addressed is: "What is the stimulator to induce hemocyte aggregation and adhesion in *Mytilus*?"

After cell-cell encounters or cell-substratum contact, hemocytes change their shapes. There must be some control system to regulate such cell behaviors. In accordance with contemporary concepts of cellular signal transduction and cell activation, these processes involve changes in the concentrations of cytosolic cAMP and/or calcium as

intracellular signals to regulate specific protein kinases that can phosphorylate specific proteins to perform specific cell function(s). The third question is therefore: "What is/are the regulation system(s) inside Mytilus hemocytes during cell activation?"

Table I-1. Characteristics of granulocytes and hyalinocytes in molluscs.

	Granulocyte	Hyalinocyte	Ref.
Pseudopodia	large pseudopodia with ribs or spikes	absent	2,5
Granules	acidophilic and/or basophilic and/or neutrophilic (0.5-1.0μm)	fine (0.2-0.4μm) and uniformly basophilic	2,4
Adhesion on glass	+++	+	4
N/C	low	high	1
Nucleus shape	small & round	large & irregular	6
Nucleoli	less prominent	large, one or two	6
Mitochondria	few	quite numerous and may form "juxtanuclear body	3
Phagocytosis	+++	+	2,4

N/C : nucleus to cytoplasmic ratio

References:

1: Bayne et al., 1979
2: Sminia, 1981
3: Hawkins & Howse, 1982
4: Cheng, 1984
5: Rasmussen et al., 1985
6: Auffret, 1988

CHAPTER II

CHARACTERIZATION OF HEMOCYTE AGGREGATION AND ADHESION AND THEIR INHIBITION IN THE CALIFORNIA MUSSEL (*Mytilus californianus*).

ABSTRACT

Within minutes of removal from the California mussel, *Mytilus californianus*, hemocytes become sticky for one another and for foreign surfaces. We sought to understand the cell surface changes responsible for this altered state. Hemocyte aggregation and adhesion assays were used in experiments in which a variety of reagents potentially capable of interfering with aggregation were screened. Caffeine, NEM, cytochalasin B, $NaIO_4$, and EDTA were completely or partially inhibitory towards aggregation and adhesion. However, sugars, RGD-containing peptides, GAGs, protease inhibitors, heparin, or poly-L-lysine were without effect. Low temperature (4°C) slowed hemocyte adhesion and hemocyte cohesion. Based on the findings, it appears that 1) *Mytilus* hemocyte aggregation, *in vitro*, is a two-step process that is mediated by temperature and requires metabolic energy and divalent cations (calcium and magnesium); 2) *Mytilus* hemocyte adhesion and hemocyte aggregation are two associated but different cell behaviors.

INTRODUCTION

Cell aggregation (cell clumping) in bivalves was first reported by Geddes (1880, cited by Narain, 1973). In molluscs, it has been presumed that clump formation is involved in wound healing (Bang, 1961; Sparks, 1972; Sminia, 1981). However, hemocyte aggregation in bivalves differs from blood clotting

in vertebrates in that no extracellular fibers are formed. The aggregation of hemocytes in bivalves is reversible, and most aggregated cells later disperse, re-entering the circulatory system as wound repair progresses (Feng & Feng, 1974).

Mussel hemocytes can spontaneously aggregate *in vitro*. When hemolymph is removed from a mussel, the free hemocytes form aggregates during the bleeding or immediately thereafter. When the aggregates and the free cells settle down on a foreign surface, they adhere (cell-substratum adhesion) and usually spread, and later migrate away. Although the phenomena of clump formation and hemocyte adhesion and spreading have been described both *in vivo* and *in vitro* in bivalves (Drew, 1910, cited by Narain, 1973; Dundee, 1953; Bang, 1961; Sparks, 1972; Sminia, 1981), few attempts have been made to unravel the operative mechanisms (Seifert, 1983). In order to study this rapid, spontaneous cell adhesion, it was first necessary to find effective inhibitors that could reversibly block the reaction. In this study, we used hemocytes of the California mussel, *Mytilus californianus*, to 1) identify inhibitors of hemocyte aggregation and adhesion *in vitro*, and then 2) investigate differences between hemocyte clump formation (cell-cell interaction) and hemocyte adhesion (cell-substratum interaction) *in vitro*.

MATERIALS AND METHODS

Chemicals

Peptides containing the Arg-Gly-Asp (RGD) amino acid sequence were kindly provided by Dr. D.W. Barnes, Biochemistry, Oregon State University. BCA protein assay reagents were purchased from Pierce. All other chemicals were purchased from Sigma Co..

The suspected inhibitors were dissolved in CMTBS (Ca^{++},

Mg^{++} Tris-bufferred saline: 10 mM $CaCl_2$, 60 mM $MgCl_2$, 50 mM Tris HCl, 960 mOsm). The EDTA was dissolved in calcium- and magnesium- free Tris-bufferred saline (CMFTBS). Hydrophobic chemicals were first dissolved in DMSO, and then mixed with CMTBS. The final concentration of DMSO in treated hemolymph was lower than 0.5 %. The pH value of solutions was adjusted to 7.4 with NaOH, and checked the osmolarity before they were mixed with hemolymph.

Animals

Individual Mytilus californianus larger than 8 cm in length were collected monthly from the rocky intertidal zone at Seal Rock State Park (15 mi south of Newport, OR). On the same day, these mussels were transferred to a filtered, recirculating, continuously aerated sea water system which was maintained at pH 7.6, nitrate < 10 ppm, nitrite < 0.2 ppm, 15°C, and close to normal salinity. Mussels were held in this system for at least 3 days before being used as a source of hemolymph.

Hemolymph collection

A plastic rod (3 mm diameter) was inserted between the two shells to prevent the shells closing, and sea water was drained from the mantle cavity. Animals were then transferred to a cold room (4°C). Hemolymph was collected from the posterior adductor muscle using a cooled sterile syringe with 18G 1½" needle. With the needle removed, the colorless (slightly opalescent) hemolymph was immediately transferred to cooled sterile tissue culture tubes (Falcon) for further treatment. Hemolymph was collected from each animal once only.

Coating of surfaces for cell adhesion assay

Except for agarose, the coating process was as follows: the coating material was loaded on the clean surface for 30 min at RT. Then the excess was washed out by CMTBS. For

agarose coating, melted agarose was added to washed 8-well slides (5 µl/well), and then mounted by a cover slip which was held off the slide by two small pieces of cover slip. The cover slips were removed after keeping the slide at 4°C for a few minutes. The thin layer of agarose did not affect microscopic observation.

Inhibitor screening assay

The solutions of suspected inhibitor were aliquoted into siliconized microtubes (PGC Scientifics, Maryland), and kept at 4°C. Slides with 8 wells (Cel-Line, NJ), cleaned by immersion in acid alcohol overnight, were dried and placed in a humidity chamber which was also kept at 4°C before use. As soon as the hemolymph had been transferred to a cooled sterile tissue culture tube, it was aliquoted and mixed (1:1) with test solutions by vortexing for about 3 seconds. The treated hemolymph was then loaded onto the undersurface of an inverted, cooled 8-well slide (50µl/well). Each sample was loaded in duplicate onto the same position on a different slide. These were maintained as hanging-drops during gyration at 100 rpm performed by Gyrotory® Shaker-Model G2 (New Brunswick Scientific Co., Inc.) at room temperature. The purpose of using hanging-drops was to avoid cell adhesion to glass during gyration. Following 15 minutes gyration, the slide was carefully turned over. Two 1mm thick spacers were placed on the slide, followed by a coverslip (24 X 50 mm). Cell aggregation, cell adhesion, and cell spreading were observed under phase contrast microscopy.

Those inhibitors which interfered with cell aggregation or cell adhesion were selected for further studies. Due to occasional individual differences between mussels, each reagent was tested on at least three hemolymph samples taken from separate mussels at different times.

Assay for inhibition of cell aggregation

This assay followed the same protocol used in the screening test. After the slide was turned so the hemolymph was on the upper side, 100 % formalin was added (1 μl/well) to fix the cells. The final concentration of formalin in hemolymph was 2 %. Cells at the center of each preparation were then immediately dispersed *in situ* by gentle pipetting five times. Samples were then mounted by a coverslip (24 X 50 mm) held on two 1mm thick spacers. The extent of cell aggregation was assessed by counting the total free cell numbers in 5 different areas in each well. Fresh hemolymph was mixed with 20 % formalin (1:1) to immediately block cell aggregation. The total (control) free cell number was counted in hemolymph fixed in 10 % formalin as soon as it was taken from the mussel.

$$\text{The percentage of cells remaining free} = \frac{\text{Total free cell numbers in test treatment}}{\text{Total free cell numbers in fixed fresh hemolymph}} \times 100$$

Assay for inhibition of cell adhesion

After hemolymph had been mixed with test solutions, this was then loaded (50 μl/well) into wells of a pre-cooled (4°C), flat-bottom 96-well tissue culture plate (Corning, New York). Each treatment was loaded in triplicate. After 15 minutes incubation at room temperature, the plasma and unattached cells were removed and each well was washed 3 times with CMTBS (100 μl/well). As an assessment of cell numbers, protein concentrations were measured by means of the BCA protein assay (Pierce). The adherent cells were first lysed in 20μl cell

lysis solution (2% Na_2CO_3, 0.1M NaOH) for at least 30 min at RT with occasional shaking by vortex, and then the fresh Pierce BCA protein assay reagent was added (100 μl/well). After 30 min incubation at 60°C, the plate was cooled to RT, and the absorbance of each well was read at 550 nm using a microtiter plate reader (Titertek Multiskan MCC/340).

The percentage of cell adhesion was calculated using the following equation:

$$[(A_i - A_b) / (A_c - A_b)] \times 100$$

A_i: Absorbance of inhibitor-treated well

A_b: Absorbance of background well (lysis solution and BCA reagent only)

A_c: Absorbance of CMTBS-treated well

According to this equation, the absorbance in background wells indicates 0 % adhesion, and the absorbance in CMTBS-treated wells indicates 100 % adhesion.

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples from different animals. Data are presented as mean ± S.D.. Statistical analysis of data was performed using paired or unpaired Student's t-test as appropriate. Differences were considered significant when $p<0.05$.

Cell viability

Following the adhesion/spreading and aggregation assay, hemocyte viability was determined by visual observation of hemocyte spreading, and by cell exclusion of propidium iodide. This test was performed only on the samples in which cell aggregation or adhesion was interrupted. Treated hemolymph (40μl) was gently removed from well slides or culture plates, and replaced by 40 μl CMTBS. If the remaining cells had not aggregated or spread in 10 minutes, two possibilities were considered; either the treated cells were killed by the chemical or the inhibitory effect of the chemical was irreversible. In order to determine whether the treated cells were alive or not, the propidium iodide staining protocol was followed: 1 μl propidium iodide (500 μg/ml in CMTBS) was added into each sample (50 μl). In cells which are dead, the dye enters and binds to nuclear DNA with high affinity. Stained nuclei generate bright red fluorescence under epi-fluorescence using a Zeiss microscope with a filter set appropriate for rhodamine isothiocyanate.

RESULTS

Normal hemocyte aggregation, adhesion, and spreading <u>in vitro</u>

When hemocytes were first removed from the mussels, they remained dispersed and were round (Fig.II-1a). However, they

changed from round to elongated in one minute or less (Fig.II-1b). Sometimes aggregation occurred extremely rapidly during the bleeding, or while the hemolymph was being dispensed into the test tube. After initial cellular contact, cells were seen to pull together before the cells or aggregates began to spread. Aggregates formed this way without shaking were small (Fig.II-1c), and the cell-cell binding strength was weak. The individual cells were distinguishable and could be easily dispersed from the aggregates by pipetting.

In contrast, clumps that formed during 10 or more minutes shaking often contained more than ten thousand cells (Fig.II-2a). This kind of clump was resistant to mechanical dispersion by pipetting or vortexing. When cells or aggregates were allowed to settle down on to glass or plastic, they attached, flattened, and developed pseudopodia. During subsequent incubation, cells located at the surface of the clump adhered to the substratum and migrated radially out of the clump (Fig.II-2b, 2c). The attached and spread cells were seen to move by amoeboid locomotion. The cells possess a strong ability to adhere, spread, and migrate on foreign surfaces. Only on agarose substrata were such kinds of cell behaviors inhibited (Table II-1).

Influences of suspected inhibitors on hemocyte aggregation, adhesion, and spreading

Based on data obtained from screening assays, the tested

reagents could be allocated into four categories. The first includes caffeine (25 mM) and N-ethyl maleimide (0.1 mM), which strongly inhibited both hemocyte aggregation and adhesion. The second category of chemicals poorly blocked hemocyte aggregation but significantly inhibited hemocyte adhesion. They include EDTA (60 mM), sodium periodate ($NaIO_4$, 10 mM), and poly-L-glutamic acid (MW 50,000-100,000; 1 mg/ml). Cytochalasin B (5 μg/ml) is the only chemical in the third category: it effectively inhibited hemocyte aggregation, but poorly blocked hemocyte adhesion. The fourth group of chemicals had no detectable inhibitory effect on either hemocyte aggregation or adhesion. They included various sugars (Table II-2), RGD- (Arg-Gly-Asp-) containing peptides, glycosaminoglycans (GAGs), poly-L-lysine, heparin, protamine, protease inhibitors, and colchicine (Table II-3). Since inhibited hemocytes could recover their aggregation and adhesion competence after caffeine, EDTA, poly-L-glutamic acid, or cytochalasin B was replaced by CMTBS, the inhibitory effects of such chemicals, unlike NEM and $NaIO_4$, were reversible (data not shown).

The extent of hemocyte aggregation can be easily distinguished at three different states, namely no aggregation, weak aggregation, and cohesive aggregation (Fig. II-3). Five different stages of hemocyte adhesion/spreading were scored (Fig.II-4).

Hemocytes aggregated and adhered in 10 mM sodium azide (NaN_3). However, when the cells were co-incubated in 10 mM NaN_3 with 25 mM caffeine for 30 min, cell aggregation and adhesion were still affected after the caffeine/azide was replaced by azide solution. Such azide-poisoned cells aggregated weakly after the NaN_3+caffeine solution was replaced by NaN_3. When the NaN_3+caffeine was replaced by CMTBS, the cells recovered eventually and formed secondary aggregates.

Low temperature (4°C) delayed cell adhesion for the first 3 min, but after 15 min no differences from controls could be seen (Fig.II-5). In cell aggregation assays, cells still aggregated weakly at 4°C. With an increase in temperature, the aggregates contracted and cohered to form tight masses as if no interruption had occurred (Fig.II-6).

Osmolarity seems not to be critical for M. californianus hemocyte aggregation and adhesion in vitro. There were no significant differences in hemocyte adhesion and aggregation when hemolymph was mixed (1:1) with CMTBS in the range 800-1100 mOsm.

The inhibitory effects of caffeine, NEM, EDTA, $NaIO_4$, and cytochalasin B on cell aggregation

Hemocytes exposed to 25 mM caffeine, 5 µg/ml cytochalasin B, or 0.1 mM NEM did not aggregate. When hemocytes were treated with EDTA or $NaIO_4$, the morphology of aggregates varied. Under the microscope, some colorless material was seen

among the aggregates. Its presence seemed to restrict hemocyte contact.

At 25 mM, caffeine completely, reversibly, and rapidly blocked hemocyte aggregation, but did not kill the cells. When the concentration of caffeine was reduced to less than 15 mM, cells formed weak aggregates. The difference between free cell numbers in 25 mM and 15 mM caffeine was statistically significant ($p<0.05$) (Fig.II-7a). Cytochalasin B (5 μg/ml) in 0.5 % DMSO resulted in 70 % of the hemocytes remaining free. DMSO (0.5 %) did not influence hemocyte aggregation and adhesion (Fig.II-7b). At 0.1 mM, NEM significantly inhibited hemocyte aggregation ($p<0.05$) (Fig.II-7c). $NaIO_4$ at 10 mM weakly inhibited hemocyte aggregation (Fig.II-7d). EDTA at concentrations 15-60 mM significantly inhibited hemocyte aggregation. However, most cells were observed forming weak aggregation, only about 20 % of cells remained free (Fig.II-7e).

The inhibitory effects of caffeine, NEM, EDTA, $NaIO_4$, and cytochalasin B on hemocyte adhesion and spreading

When hemolymph was treated with 5 μg/ml cytochalasin B, 0.1 mM NEM, or 10 mM $NaIO_4$, the hemocytes remained round, thus resembling naive cells. Cytochalasin B (1 μg/ml) could retard hemocyte spreading, affect the morphology of spread hemocytes, and inhibit hemocyte migration. Cells exposed to 25 mM caffeine were irregular in outline (Fig.II-8a). The 0.6-60 mM

EDTA inhibited cell adhesion, but did not abolish spike formation (Fig.II-8b).

The concentration of caffeine necessary to inhibit cell adhesion was similar to that needed to inhibit aggregation. At 15 mM caffeine, cell adhesion was significantly greater relative to that in 25 mM caffeine (Fig.II-9a). Cytochalasin B significantly inhibited cell adhesion at concentrations between 1 μg/ml and 5 μg/ml ($p<0.05$), though 60 % of the cells were still adherent in 5 μg/ml cytochalasin B treatment (Fig.II-9b). The inhibitory activity of NEM (0.01 mM to 10 mM) was significant (Fig.II-9c). Concentrations in this range inhibited 90 % of cell adhesion on culture plates (polystyrene). Both $NaIO_4$ (10 mM) and EDTA (≥ 0.6 mM) influenced cell adhesion (Fig.II-9d & 9e). When EDTA-treated hemolymph was replaced by 20 mM or higher concentration of buffered isotonic Ca^{++} or Mg^{++} solution, 80 % of hemocytes recovered their adhesion competence (Fig.II-10). Therefore, Mytilus hemocyte adhesion is Ca^{++} and/or Mg^{++}-dependent.

DISCUSSION

A variety of buffers have been used in mussel hemocyte studies (Moore & Lowe, 1977; Renwrantz et al., 1985; Dageförde et al., 1986). Although the buffer osmolarity can affect phagocytic activity in M. edulis (Renwrantz, 1989), pH in the

range 7.4 to 8.4 or osmolarity in the range 800 mOsm to 1050 mOsm did not interfere with hemocyte aggregation in studies reported here (data not shown). To resemble the sea water in which the mussels were held, a pH of 7.4 and salt content of 960 mOsm were selected for the CMTBS.

Several lectins (carbohydrate specific binding proteins) have been isolated from invertebrates (Sharon & Lis, 1972; Lackie, 1980; Yeaton, 1981; Vasta et al., 1982). Although they are undoubtedly involved in cell recognition (Sminia et al., 1979; van der Knaap et al., 1982; Fryer et al., 1989), the full range of their functions remains to be understood (Yeaton, 1981; Brandly & Schnaar, 1986). Like those in slime molds (Sadeghi & Klein, 1988), molluscan lectins have been suspected to be functionally associated with cell aggregation and adhesion (Renwrantz et al., 1985). $NaIO_4$ is a reducing agent and can open the ring structure of carbohydrates. In this study, $NaIO_4$ was found to interefere with hemocyte adhesion and aggregation. It can be inferred that carbohydrates on hemocyte surfaces are probably involved in this kind of cell-cell and cell-matrix binding. However, about 50 different kinds of sugars were tested for inhibitory activity in this study. None was effective. Sugar structures on epithelial cell surfaces have been demonstrated that are important to bacterial adhesion and invasion (Finlay et al., 1989). These adhesion molecules may recognize specific attributes of complex carbohydrate structures, not single

carbohydrate residues (Brandley & Schnaar, 1986). The absence of adhesiveness in naive cells could be due to the effects of surface charge. Most cells are negatively charged on their surfaces (often by sialic acids), and the resulting repulsive forces between blood cells have been postulated to separate cells in their naive state (Bell, 1983). Cell activation may reduce the negative surface charge by loss of sialic acids (Rutishauser et al., 1988). In order to evaluate possible charge effects on cell aggregation and adhesion, heparin and protamine were added to cell suspensions. All results were negative (data not shown). Heparin is widely used to inhibit platelet clotting, however, it did not affect hemocyte aggregation in this study, in accordance with results in insects (Ratner & Vinson, 1983) and Limulus (Bryan et al., 1964). In this study, hemocytes adhered on differently treated surfaces except on 1 % agarose surface. Agarose has been used to inhibit cell adhesion of various cell types; the inhibitory mechanism is unknown.

In both platelet clotting (Guyton, 1986) and the prophenoxidase (proPO) system of arthropods (Lackie, 1988; Johansson & Söderhäll, 1989), serine-protease cascade reactions have been implicated. These reactions are controlled by various protease inhibitors (Hergenhahn et al., 1987). Protease inhibitors can affect hemocyte phagocytosis also (Fryer et al., 1991). However, protease inhibitors tested here failed to block mussel hemocyte aggregation or adhesion.

Both GAGs and RGD-containing peptides are involved in cell-substratum and cell-cell interactions (Underhill, 1982; Roseman, 1985). Furthermore, a sponge aggregation factor has been revealed that is a sulfated polysaccharide (Coombe & Perish, 1988). However, GAGs are not involved in mouse blastocyst (Morris & Potter, 1984). In hemocyte aggregation and adhesion test, none of the GAGs or RGD peptides tested in this study had any significantly inhibitory effect except for some retardation of hemocyte spreading.

N-ethyl-maleimide has been reported to irreversibly inhibit the aggregation of horseshoe crab hemocytes (Bryan et al., 1964). Similarly, NEM interfered with Mytilus hemocyte aggregation and adhesion. Thus, it can be inferred that the normal disulfide bond structures of proteins maintain protein functions and regulate these two hemocyte behaviors.

It has been reported that the inhibition of cytoskeleton assembly disrupts plasmatocyte encapsulation (Davies & Preston, 1987), hemocyte chemotaxis (Cheng & Howland, 1982), and cell migration, as well as cell surface receptor redistribution (Cheng & Howland, 1982; Dageförde et al., 1986). Since bivalve hemocytes alter their shapes in both cell aggregation and cell adhesion (Jones & Gillett, 1976; Feng, 1988), it is inferred that the cytoskeleton plays an important role in these two processes. Cytochalasin B and colchicine have been widely used to reduce the rate of actin polymerization into microfilaments and the assembly of

microtubules from monomeric tubulins, respectively. In this study, cytochalasin B was more inhibitory towards hemocyte aggregation than towards hemocyte adhesion. It also retarded hemocyte spreading, and restricted hemocyte migration. These data imply that actin microfilaments are involved in shape change during cohesion. Hemocyte spreading and migration are actin-dependent, but hemocyte adhesion may not be associated with actin.

Caffeine has been used as an anticoagulant in slime molds (Brenner & Thoms, 1984) and in several invertebrates (Bertheussen & Seljelid, 1978; Ratner & Vinson, 1983), but its inhibitory mechanism is not clear. The pharmacological effect of caffeine is known to be due to phosphodiesterase inhibition and the consequent increase in intracellular cAMP. In M. californianus, inhibition by caffeine is a dose-dependent, reversible reaction which does not affect cell viability. Suspicions that the inhibitory effect of caffeine on cell aggregation and adhesion is receptor-associated were based on microscopic observations. Hemocytes treated with caffeine for 30 min were able to recover their aggregation and adhesion competences immediately when caffeine was replaced by CMTBS. And previously spread hemocytes rounded up immediately when they were exposed to caffeine. If the inhibitory targets of caffeine were inside the cells, the cell behaviors would not be affected by caffeine so quickly.

Cell spike formation has been shown to be involved in both platelet aggregation (Born, 1962) and slime mold aggregation (Garrod & Born, 1971). However, in this study, some EDTA-treated hemocytes formed spikes, but still remained free. Similar results were reported with easten oyster (Fisher, 1986) and limpet hemocytes (Davies & Partridge, 1972). Therefore, spike formation seems not to be causally related to aggregation in these molluscan hemocytes.

It is generally recognized that Ca^{++} and/or Mg^{++} are important in ligand-receptor binding and in maintaining normal functions of various cell adhesion molecules (Müller, 1980; Springer, 1990). In hemocyte aggregation studies, Ca^{++} and/or Mg^{++} have been found to be essential for normal cell behaviors (Davies & Partridge, 1972; Kenney et al., 1972; Jumblatt et al., 1980; Kanungo, 1982; Shozawa & Suto, 1990). For M. californianus hemocytes, 0.6 mM EDTA could completely block hemocyte adhesion. Normally, mussel hemolymph contains 10 mM calcium, 60 mM magnesium and trace amounts of other divalent cations (Bayne et al., 1976). The 0.6 mM EDTA chelating capacity apparently did not remove all divalent cations from mussel hemolymph. However, its inhibitory effect was significant. It has been suggested that EDTA may not only serve as chelator, but also influence membrane permeability to divalent cations (Kenney et al., 1972; Kanungo, 1982). In the present studies, EDTA-treated hemocytes aggregated weakly, and the inhibitory effect of EDTA on cohesive aggregation

could be overcome by Ca^{++} or Mg^{++} supplements. Therefore, Mytilus hemocyte aggregation may proceed through two sequential stages. Weak aggregation is the early stage, and is Ca^{++}/Mg^{++}-independent. Cohesive aggregation is the late stage, and is Ca^{++}/Mg^{++}-dependent. Similar results were described for hemocyte aggregation in limpets (Davies & Partridge, 1972), and in Limulus (Kenney et al., 1972), as well as for coelomocyte aggregation in holothurians (Fontaine & Lambert, 1977), and in sea stars (Kanungo, 1982). All of these hemocyte or coelomocyte aggregations are two-stage reactions. One is Ca^{++}/Mg^{++}-independent, and the other is Ca^{++}/Mg^{++}-dependent.

Hemocyte aggregation in Mytilus was retarded by low temperature (4°C). Similarly, low temperature affected hemocytes in Limulus (Kenney et al., 1972), and in limpets (Davies & Partridge, 1972).

In summary, Mytilus hemocyte aggregation is a two-step process which is retarded at low temperature, and requires endogenous metabolic energy and divalent cations. Based on these results and on aggregation studies in several other cell models (Davies & Partridge, 1972; Jumblatt et al., 1980; Kanungo, 1982; Gibralter & Turner, 1985; Pizzey et al., 1988), two-step cell aggregation mediated by divalent cations and temperature appears to have been well conserved during evolution.

A major goal of this study was to characterize distinctive features of self recognition (hemocyte-hemocyte binding) and non-self recognition (hemocyte-matrix binding). Since no aggregation-specific inhibitor was found, these results do not constitute strong evidence that cell aggregation and adhesion are two independent cell activities. However, some observations imply that this is likely. First, higher concentrations of inhibitor are necessary to block hemocyte aggregation than to affect hemocyte adhesion. Second, when reversible inhibitors such as caffeine and EDTA were removed or diluted, hemocyte aggregation occurred earlier than hemocyte adhesion.

Figure II-1. The sequential processes of Mytilus californianus hemocyte aggregation In Vitro.

Figure II-1a. Naive free cell stage. These fresh hemocytes are approximately spherical in outline. (Magnification = 350 X)

Figure II-1b. Initial stage of cell aggregation and adhesion. Within one minute, all of the cells form spikes, and some cells form small aggregates (cell number < 10). (Magnification = 350 X)

Figure II-1c. Medium sized clumps. When these small aggregates collide, they can form bigger clumps. Each medium sized clump may be composed of about one hundred cells. (Magnification = 200 X)

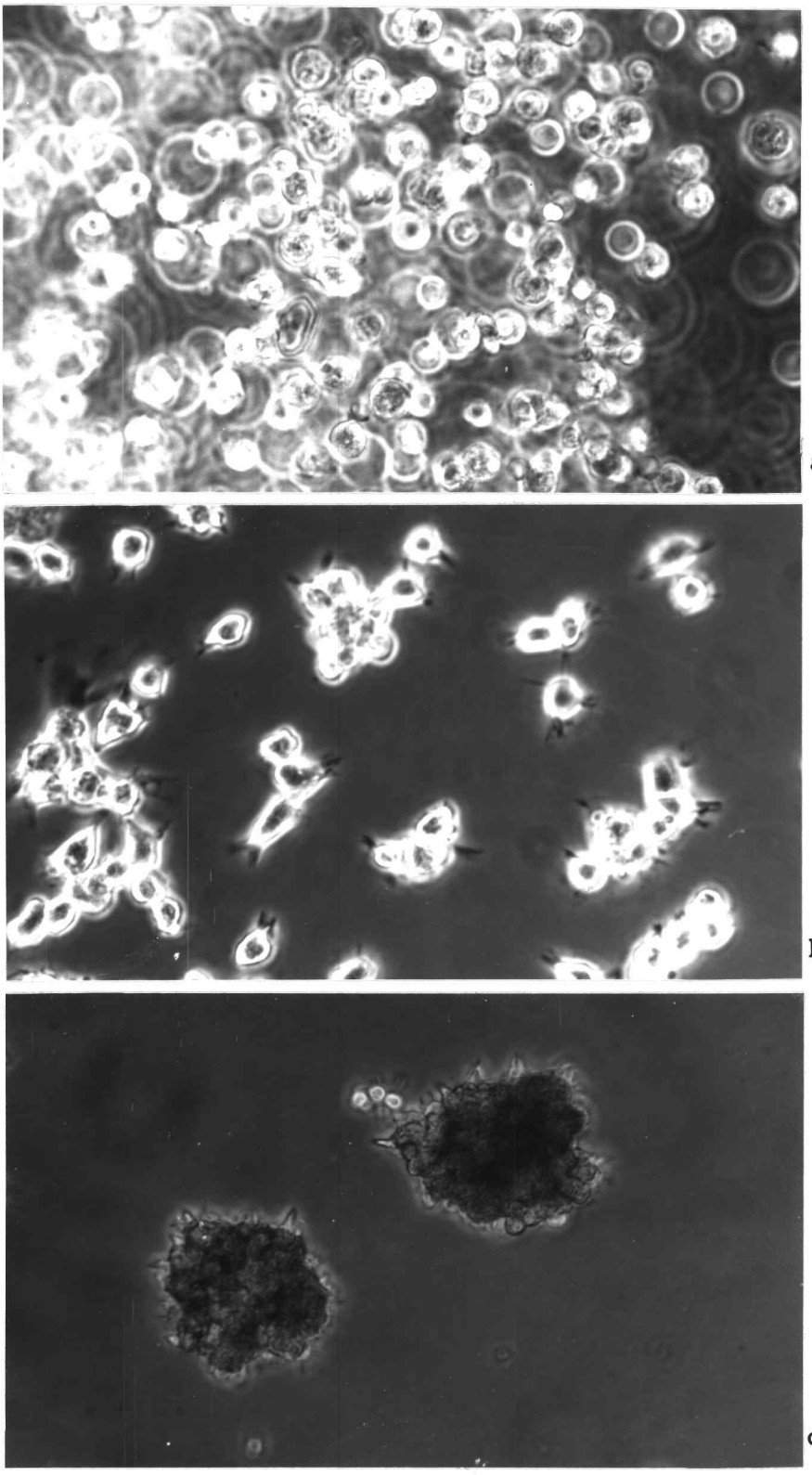


Figure II-1

Figure II-2. *Mytilus californianus* hemocyte spreading and migration *In Vitro*.

Figure II-2a. Part of a large clump. Each large aggregate like this may contain more than ten thousand cells. Cells tightly cohere together, and the individual cells are difficult to distinguish. (Magnification = 350 X)

Figure II-2b. Early stage of hemocyte migration. Cells at the edge move out of the aggregate 3 minutes after the aggregate settles down. (Magnification = 350 X)

Figure II-2c. Late stage of hemocyte migration. Cells radially migrate from the aggregate after 10 min *in vitro*. (Magnification = 120 X)

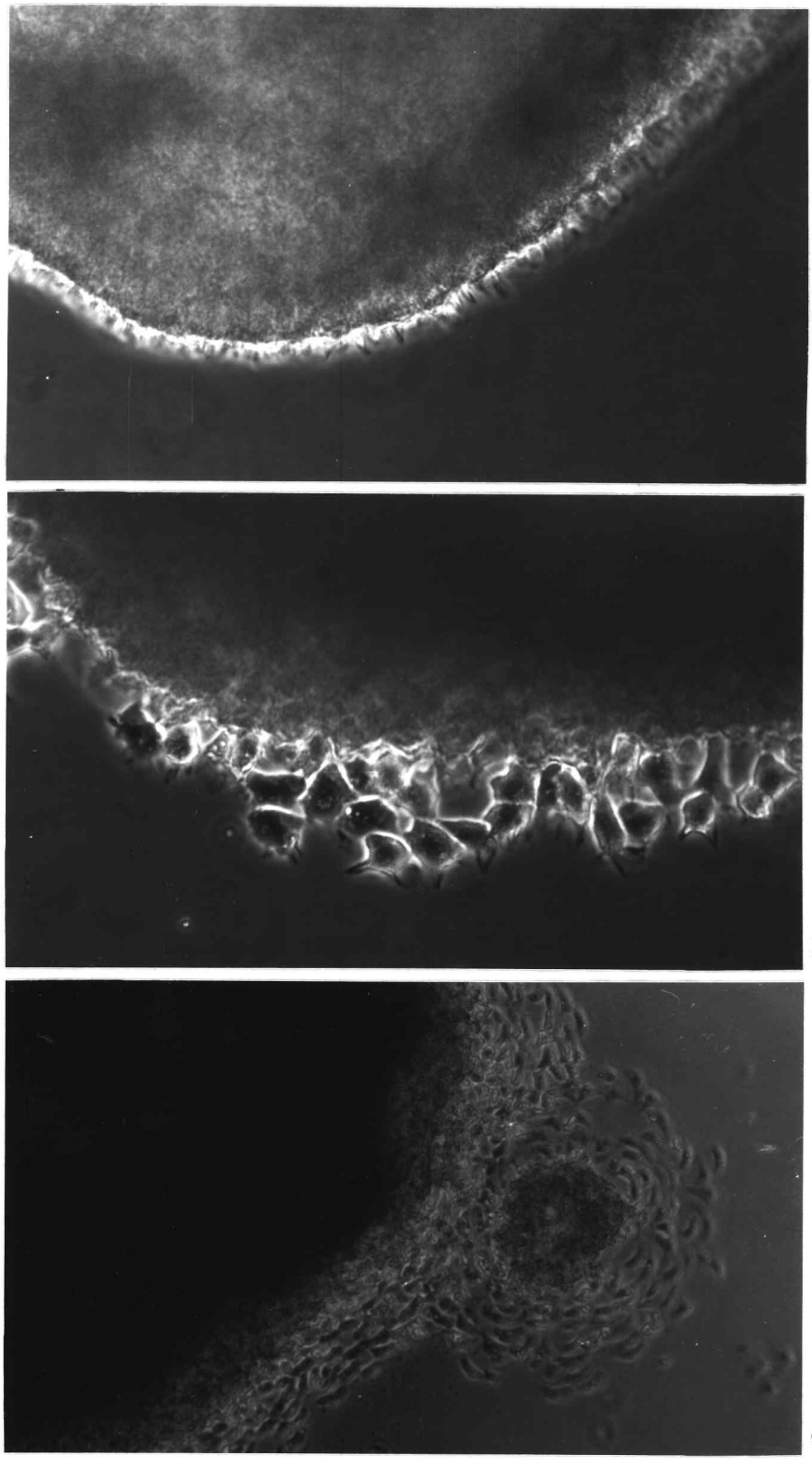


Figure II-2

Table II-1. *Mytilus californianus* hemocyte behavior on various surfaces. Fresh hemolymph was loaded onto different surfaces. After 15 minutes incubation at RT, the non-adherent cells were removed, and then cell adhesion, spreading, and migration were observed under a phase contrast microscope.

Surface	Adhesion	Spreading	Migration
glass (well slide)	+++	+++	+++
polystyrene (PS) (culture plate)	+++	+++	+++
Sigmacoted® glass	+++	+++	+++
siliconized PS	+++	+++	+++
poly-L-lysine coated (1mg/ml) glass and PS	+++	+++	+++
poly-glutamate coated (1mg/ml) glass and PS	+++	+++	+++
BSA (1%) coated glass and PS	+++	+++	+++
fibronectin (1%) coated glass and PS	+++	+++	+++
1% agarose coated glass	–	–	–

PS: Polystyrene

BSA: Bovine serum albumin

+++: strong; ++: medium; +: weak; -: inhibition

Table II-2. Sugars screened for their ability to influence hemocyte aggregation and adhesion.

Monosaccharides	
Pentoses	D(-) arabinose, L(+) arabinose, D(-) ribose, D(+) xylose, α-deoxyl-D-ribose.
Hexoses	D(+) digitoxose.
Gluc-	α-D(+) glucose, β-D(+) glucose, D-glucose-6-phosphate ($2Na^+$), D-glucuronic acid (Na^+), D(+) glucosamine-HCl, D-glucitol(D-sorbitol) D-glucuronic acid lactone (glucuronolactone), glucopyranoside (salicin), 3-O-methyl-glucopyranose, α-D(+)-glucose pentaacetate*, glucose-1-phosphate, N-acetyl-glucosamine.
Galac-	D(+) galactose, galactosamine-HCl, galactol* (dulcitol), galacturonic acid, α-deoxyl-D-galactose, 6-deoxyl-L-galactose (L-fucose), 1-O-methyl-α-D-galactopyranoside, N-acetyl-galactosamine.
Fruct-	β-D(-) fructose.
Mann-	L(-) mannose, D(+) mannose, D-mannosamine-HCl D-mannitol, α-methyl-D-mannoside, 6-deoxyl-L-mannose (α-L-rhamnose), mannose-6-phosphate.
Disaccharides	D(+) cellobiose, palatinose, β-gentiobiose, α-D(+) melibiose, α-lactose, maltose, sucrose, trehalose.
Trisaccharides	D(+)-melezitose.
Oligosaccharides	stachyose, D(+) raffinose.
Polysaccharides	chitin*(1%), laminarin(1%).

Sugars were dissolved in CMTBS, and the sugar solutions then were adjusted pH to 7.4, and mOsm to 960.

* : difficult to dissolve in CMTBS

Table II-3. Chemicals tested for inhibition of aggregation and adhesion of *Mytilus californianus* hemocytes.

Chemicals (final concentration)	
Protease Inhibitors*	phenylmethylsulfonylfluoride (PMSF) (20 μg/ml), soybean trypsin inhibitor (20 μg/ml), pepstatin A (10 μg/ml), aprotinin (10 μg/ml), leupeptin (10 μg/ml), α_2-macroglobulin (1 mg/ml).
RGD Peptides (2.5 mg/ml)	RGDS, GRGDSP, GDSP, TGRG.
Glycosaminoglycans (1 mg/ml)	hyaluronic acid, chondroitin sulfate (type A, type C).
Others	heparin (10 unit/ml), protamine (1 mg/ml), poly-L-lysine (1 mg/ml), poly-L-glutamic acid (1 mg/ml).

* PMSF, pepstatin A, aprotinin, and leupeptin were dissolved in DMSO. The final concentration of DMSO was less than 0.5%.

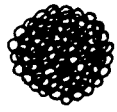
Hemocyte Aggregation			
State	no aggregation	weak aggregation	cohesive aggregation
Model			

Figure II-3. The appearances of Mytilus californianus hemocytes at three states of aggregation in vitro.

The aggregation of California mussel hemocytes in vitro can be categorized into three levels, namely no aggregation, weak aggregation, and cohesive aggregation. No aggregation (0°): Cells accumulate at the central area of the well as a result of the gyratory force. A few small aggregates are sometimes seen, while in most cases the centralized cells just pile up, and no binding occurs between them. Such cells can be freely dispersed by gentle pipetting. Weak aggregation (1°): Individual cells are distinguishable, especially those located at the edge of the aggregate. Large clumps like this can be separated by pipetting to yield several small aggregates. Due to the presence of mucous material (which is induced by certain inhibitors such as EDTA, $NaIO_4$), hemocyte contacts are sometimes restricted, and the morphology of weak aggregation varies. Cohesive aggregation (2°): The compact nature of such aggregates prevents dispersion by pipetting or vortexing. The aggregated cells cannot be seen individually.

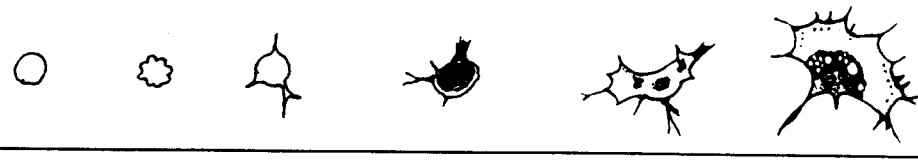
Hemocyte Adhesion/Spreading					
Stage	0°	1°	2°	3°	4°
Model					

Figure II-4. Illustration of the stages of <u>Mytilus californianus</u> hemocyte adhesion, and spreading <u>in vitro</u>.

Hemocyte adhesion and spreading are continuous processes which can be divided into five stages from 0° to 4°. Normally, hemocytes <u>in vitro</u> can adhere and spread well (4°) in 5-10 minutes. 0°- illustrates naive hemocytes in suspension which resemble cells in which adhesion and spreading are inhibited; 1°- Cell adhesion and spreading are blocked, but spike formation is not; 2°- Cells adhere and flatten, but without extension of pseudopodia; 3°- Cells extend small pseudopodia as the cells spread.

Figure II-5

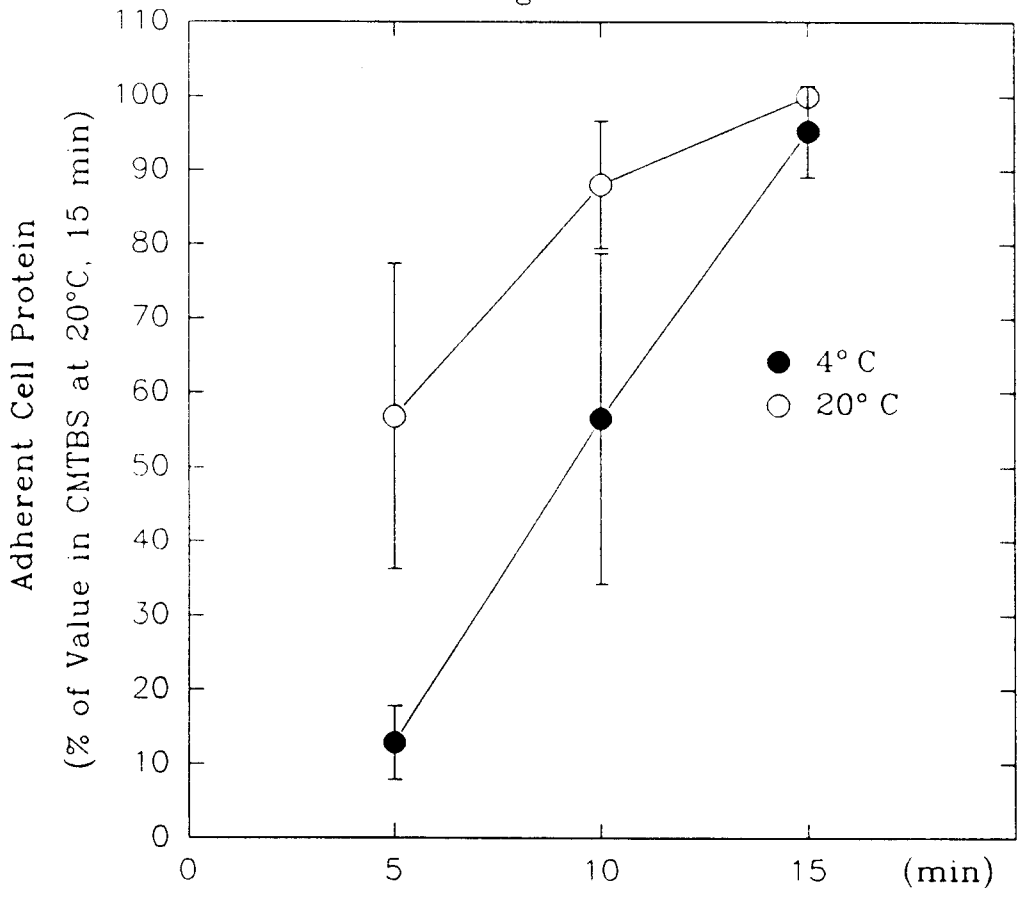
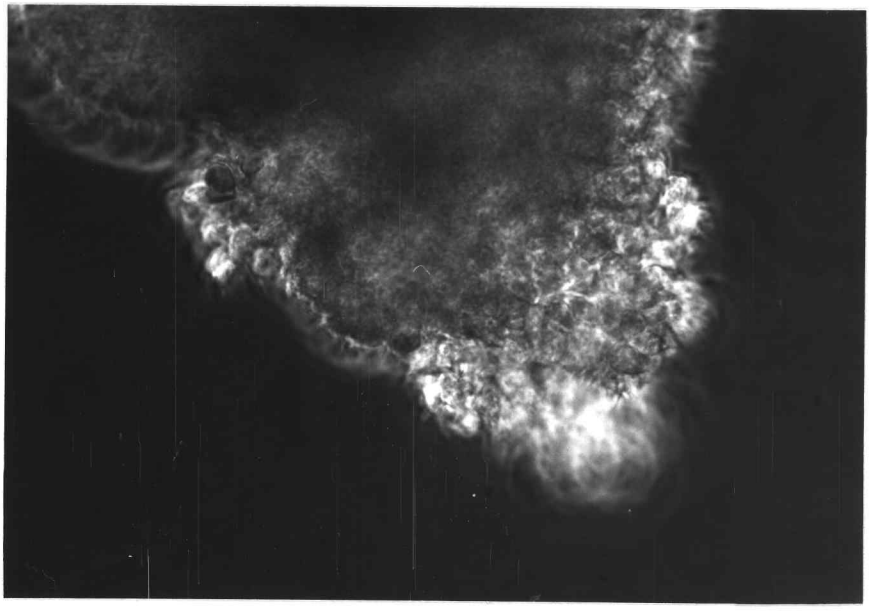


Figure II-5. The influence of temperature on the adhesion of <u>Mytilus californianus</u> hemocytes <u>in vitro</u>. Hemolymph was loaded into 96-well culture plates (50 µl/well). These were then incubated at 4°C or RT. At 5, 10, and 15 min, the non-adherent cells and plasma were removed. The wells were washed three times with CMTBS, and then the adherent cells were lysed for BCA protein assay. The values of protein concentrations obtained from RT, 15 min incubation were used as 100% hemocyte adhesion. There is a significant difference ($p<0.05$) after 5 min incubation.

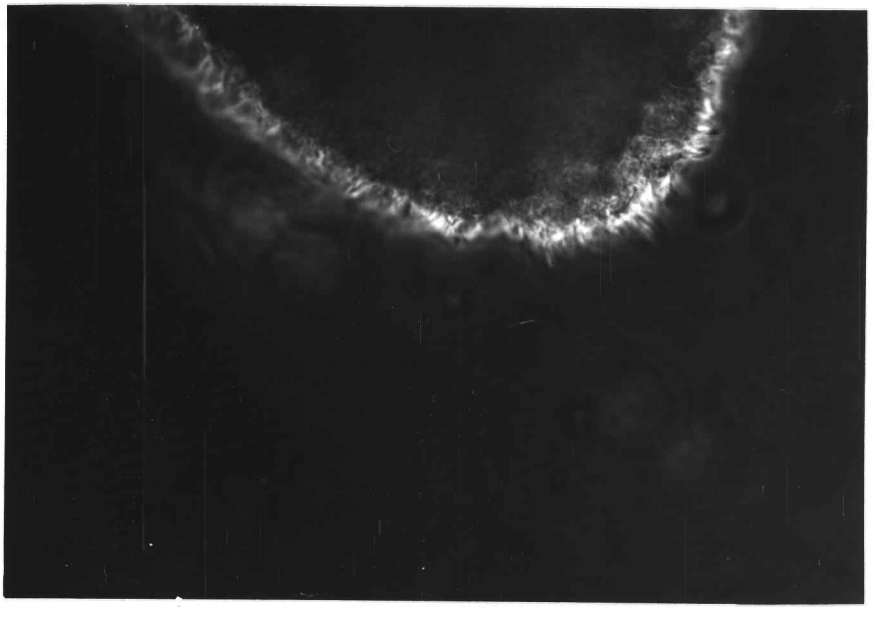
Figure II-6. The effect of temperature on *Mytilus californianus* hemocyte aggregation *in vitro*.

Figure II-6a. Weak aggregates like this one formed by 15 minutes gyration at 4°C. This photo was taken immediately after the aggregate was removed from 4°C. (Magnification = 350 X)

Figure II-6b. Cohesive aggregates formed after the incubation temperature was warmed up to RT. The whole cell mass gradually shrunk when the incubation temperature increased. This contraction led to the formation of a tighter clump. Photograph 6a and 6b were taken from the same microscopic field at 1 and 15 minutes respectively. (Magnification = 350 X)



a)



b)

Figure II-6

Figure II-7. The influence of caffeine (a), cytochalasin B (b), NEM (c), $NaIO_4$ (d), and EDTA (e) on *Mytilus californianus* hemocyte aggregation *in vitro*. In these figures, each value (%) is the ratio of free cell number in inhibitor solution to free cells in 10 % formalin.

Figure II-7a

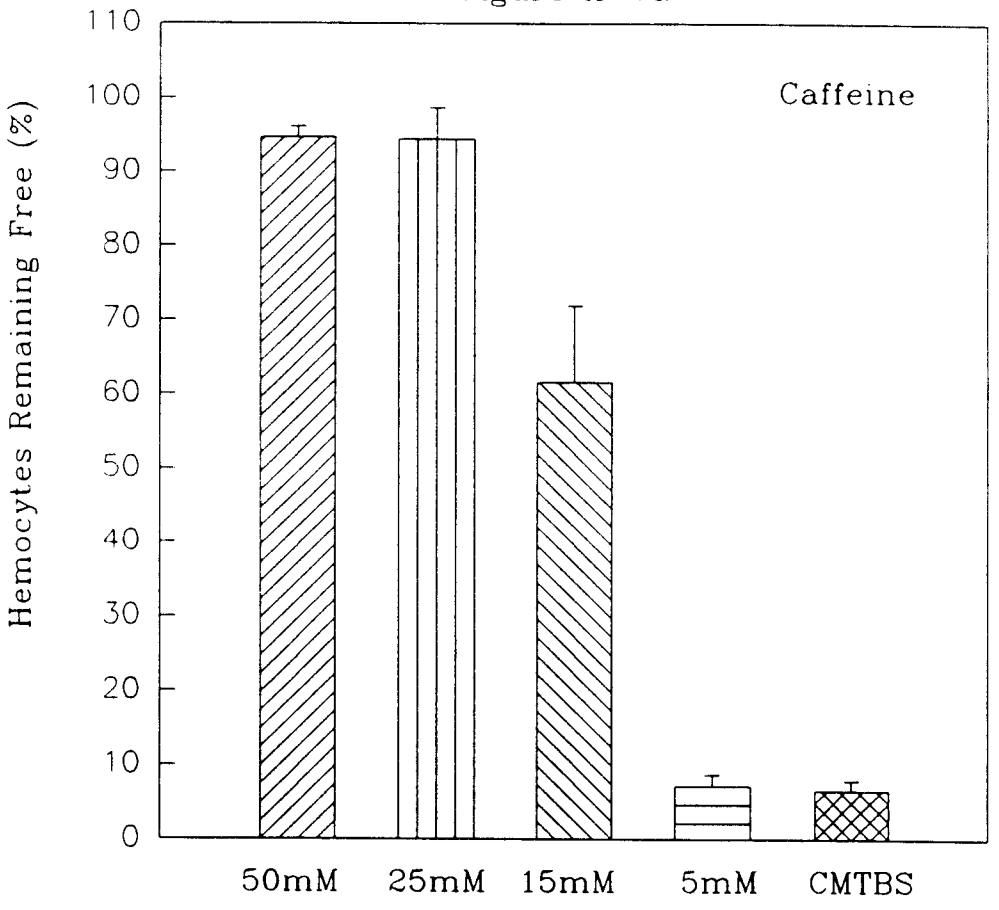


Figure II-7a. Inhibitory effect of caffeine on <u>Mytilus</u> hemocyte aggregation <u>in vitro</u>. At 25 mM or 50 mM, caffeine completely blocked hemocyte aggregation, thus most cells remained free. At 15 mM, caffeine still significantly inhibited hemocyte aggregation ($p < 0.05$). Like saline (CMTBS), 5 mM caffeine had no inhibitory effect.

Figure II-7b

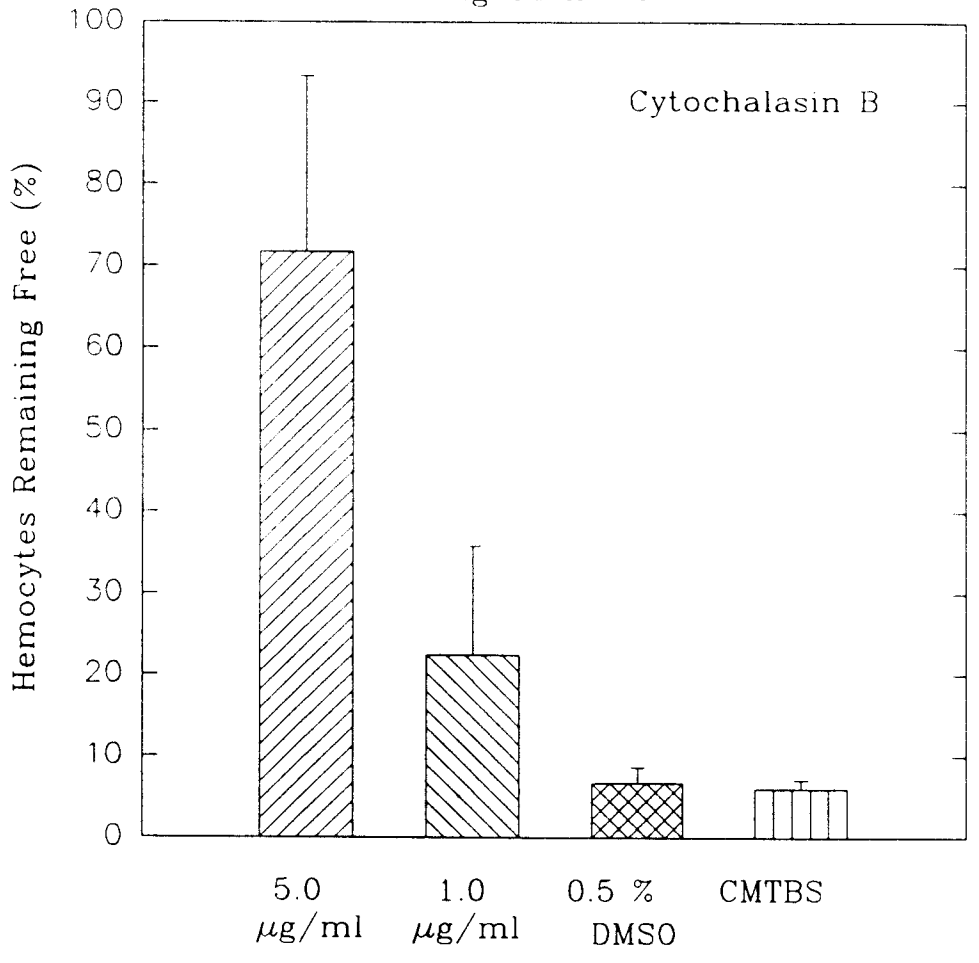


Figure II-7b. Inhibitory effect of cytochalasin B on <u>Mytilus</u> hemocyte aggregation <u>in vitro</u>. Cytochalasin B (5 μg/ml in 0.5% DMSO) significantly inhibited hemocyte aggregation ($p < 0.05\%$). The vehicle, DMSO, did not have any inhibitory effect.

Figure II-7c

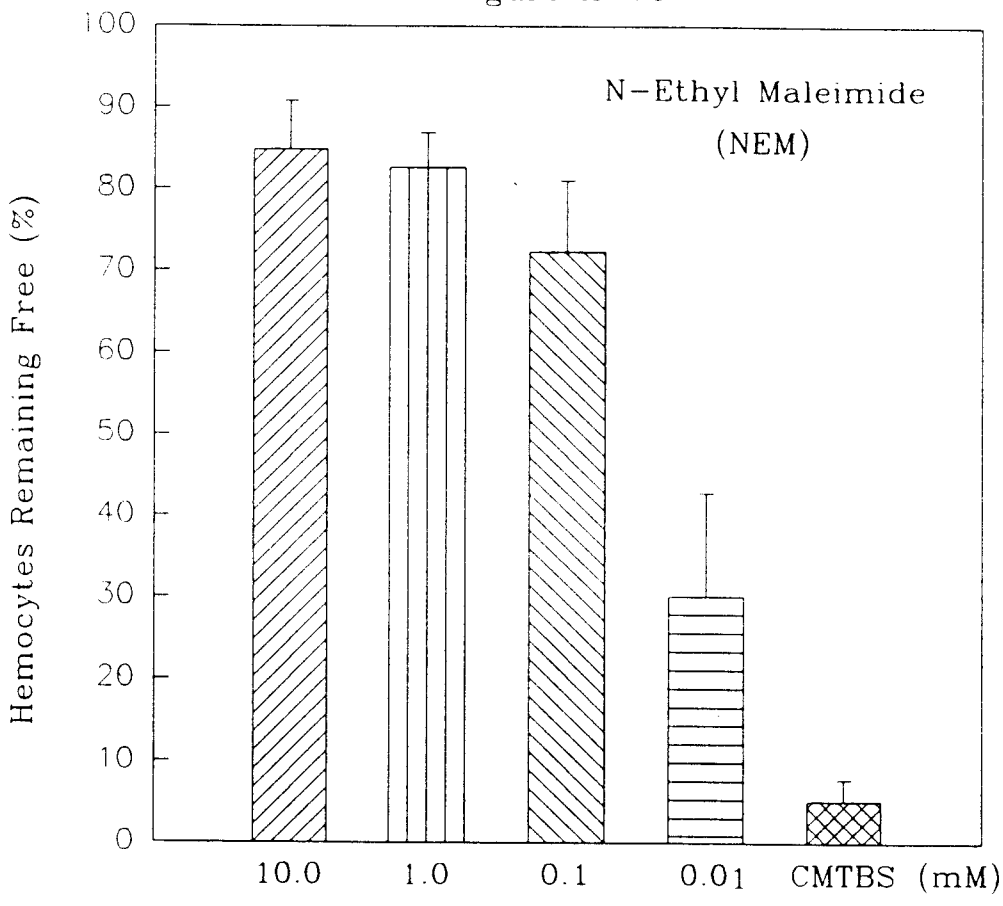


Figure II-7c. Inhibitory effect of NEM on <u>Mytilus</u> hemocyte aggregation <u>in</u> <u>vitro</u>. Hemocyte aggregation was strongly inhibited by 0.1 mM or higher concentrations of NEM ($p<0.05$).

Figure II-7d

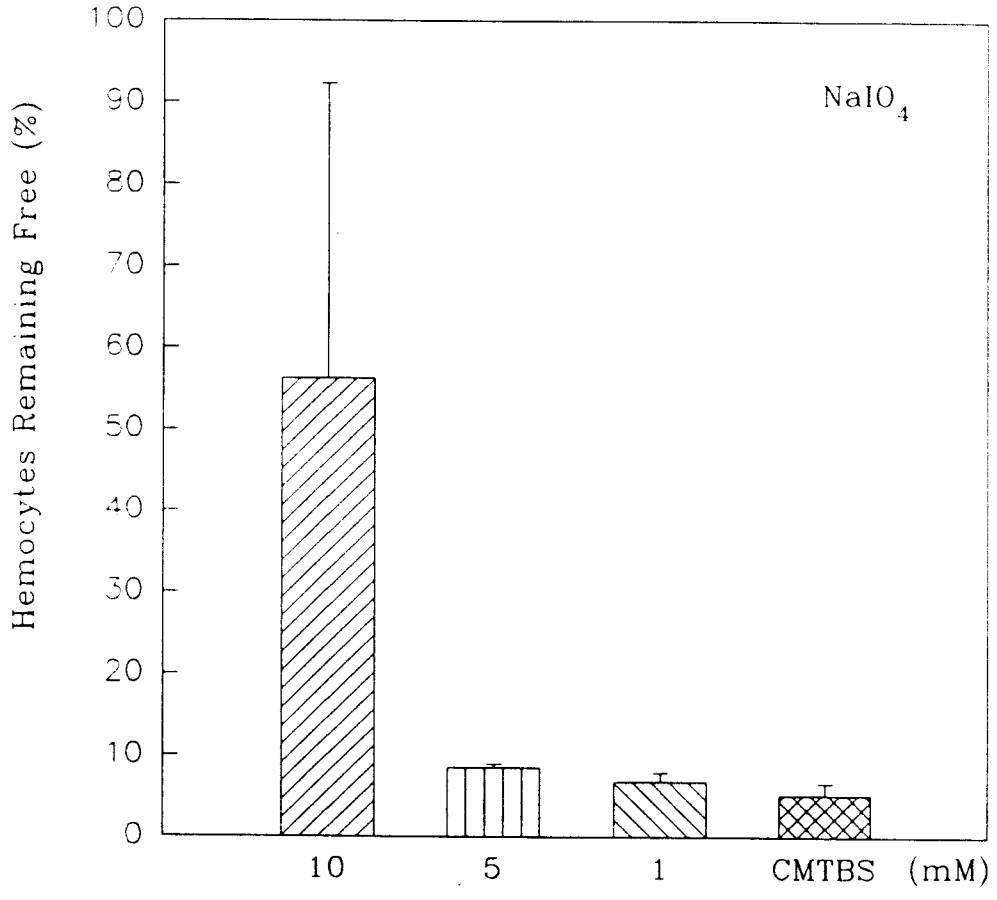


Figure II-7d. Inhibitory effect of $NaIO_4$ on <u>Mytilus</u> hemocyte aggregation <u>in vitro</u>. For sodium periodate ($NaIO_4$), 10 mM is a critical concentration for influence on hemocyte aggregation ($p<0.05$). At 5 mM or less, $NaIO_4$ did not block hemocyte aggregation.

Figure II-7e

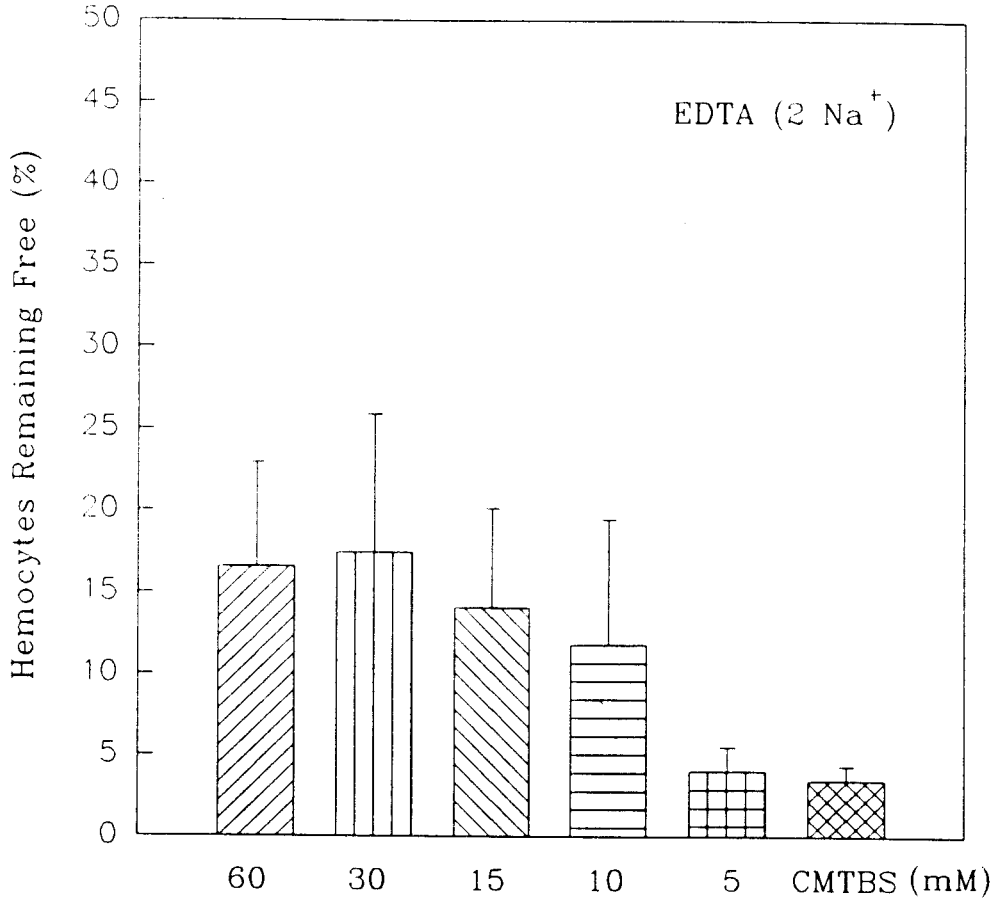


Figure II-7e. Inhibitory effect of EDTA on <u>Mytilus</u> hemocyte aggregation <u>in vitro</u>. EDTA within the range of concentrations from 15 mM to 60 mM have significant influence on hemocyte aggregation ($p<0.05$).

Figure II-8. The morphology of inhibitor-treated Mytilus californianus hemocytes in vitro.

Figure II-8a. The morphology of caffeine-treated hemocytes. The irregular shape is the main characteristic of hemocytes which were treated by caffeine (25mM). Such cells gradually become spherical over 30 minutes. (Magnification = 350 X)

Figure II-8b. The morphology of EDTA-treated hemocytes. This is a part of cell clump in 60 mM EDTA (pH 7.4). Spike formation is a common phenomenon in EDTA-treated hemocytes (arrows). These cells could not further spread when the EDTA concentration was above 0.6 mM. (Magnification = 350 X)

Figure II-8c. The morphology of $NaIO_4$-treated hemocytes. The $NaIO_4$-treated hemocytes remained round. Such cell morphology was also observed in NEM- or cytochalasin B-treated cells. (Magnification = 200 X)

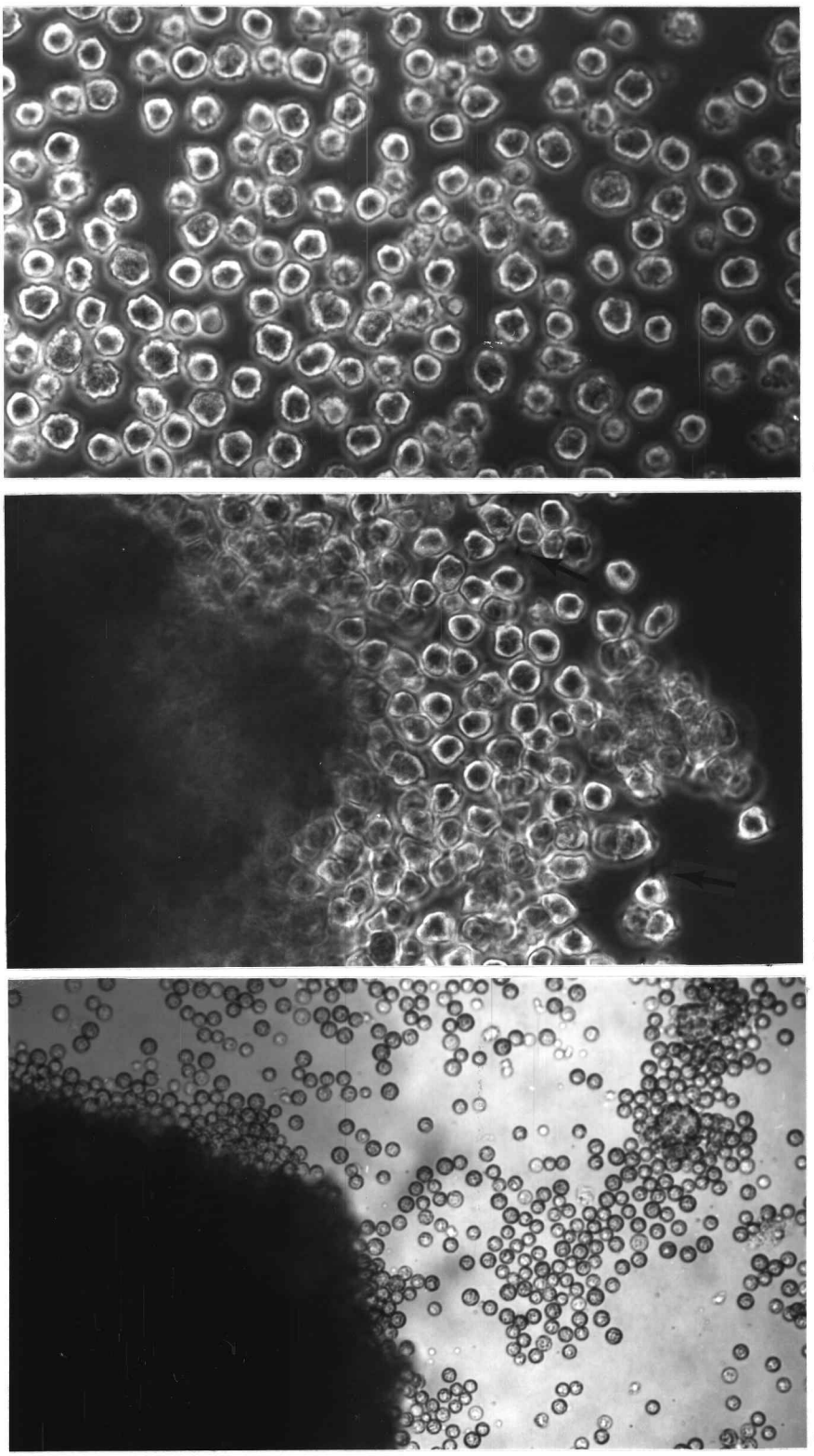


Figure II-8

Figure II-9. The influences of caffeine (a), cytochalasin B (b), NEM (c), $NaIO_4$ (d), and EDTA (e) on M. californianus hemocyte adhesion in vitro. In these figures, each bar represents (%) the ratio of the protein value of adherent cells in inhibitor solution to the equivalent value in CMTBS. That is, CMTBS-mixed hemocyte adherence is taken as 100 % adhesion competence.

Figure II-9a

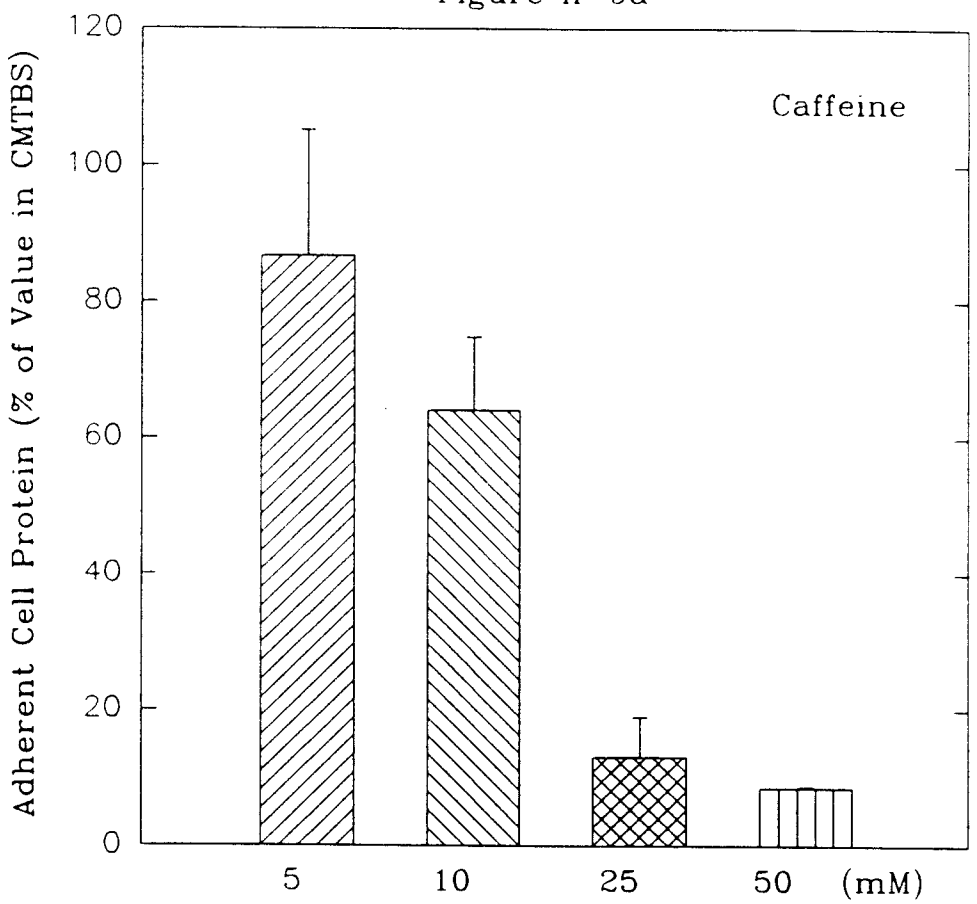


Figure II-9a. Inhibitory effect of caffeine on <u>Mytilus</u> hemocyte adhesion <u>in vitro</u>. At 25 mM or higher, caffeine significantly blocked hemocyte adhesion ($p<0.05$). At 10 mM, caffeine was still partially inhibitory.

Figure II-9b

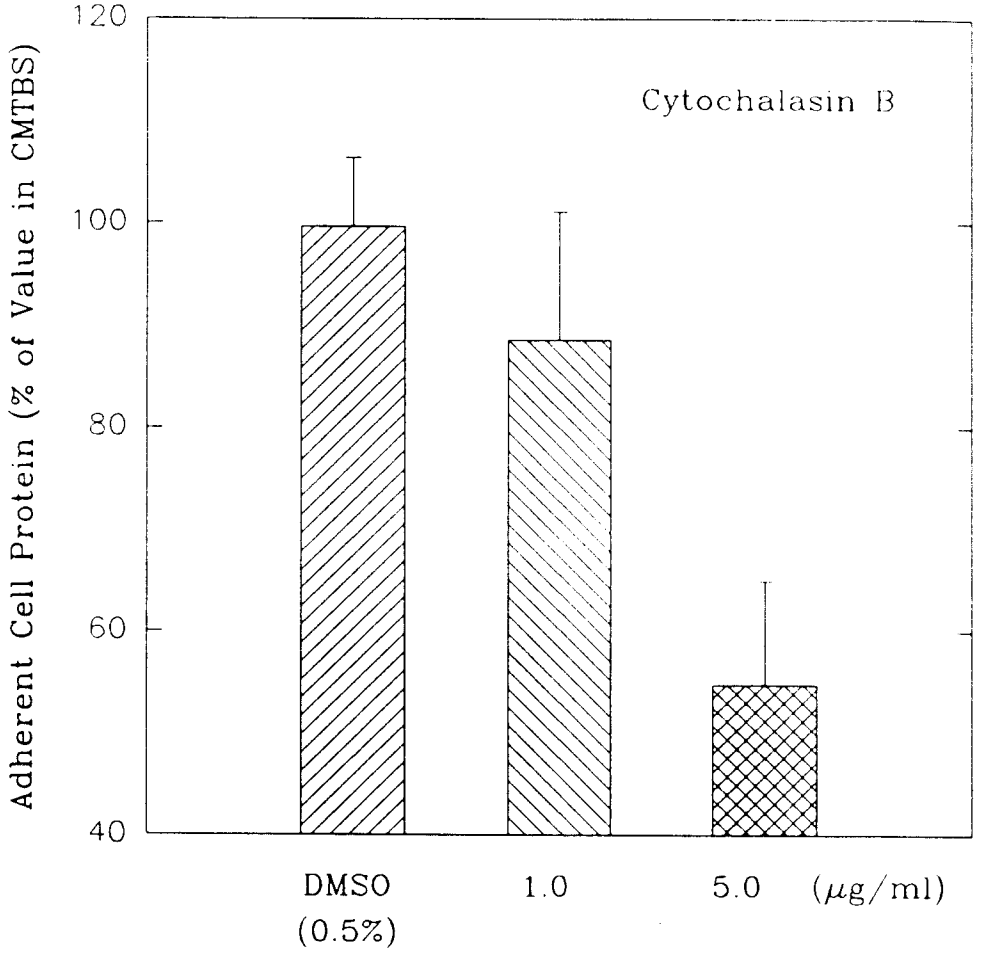


Figure II-9b. Inhibitory effect of cytochalasin B on <u>Mytilus</u> hemocyte adhesion <u>in</u> <u>vitro</u>. Cytochalasin B (5 μg/ml in 0.5% DMSO) significantly inhibited hemocyte adhesion ($p<0.05$), but about 55 % of hemocytes remained adherent. The vehicle, DMSO, did not have any inhibitory effect.

Figure II-9c

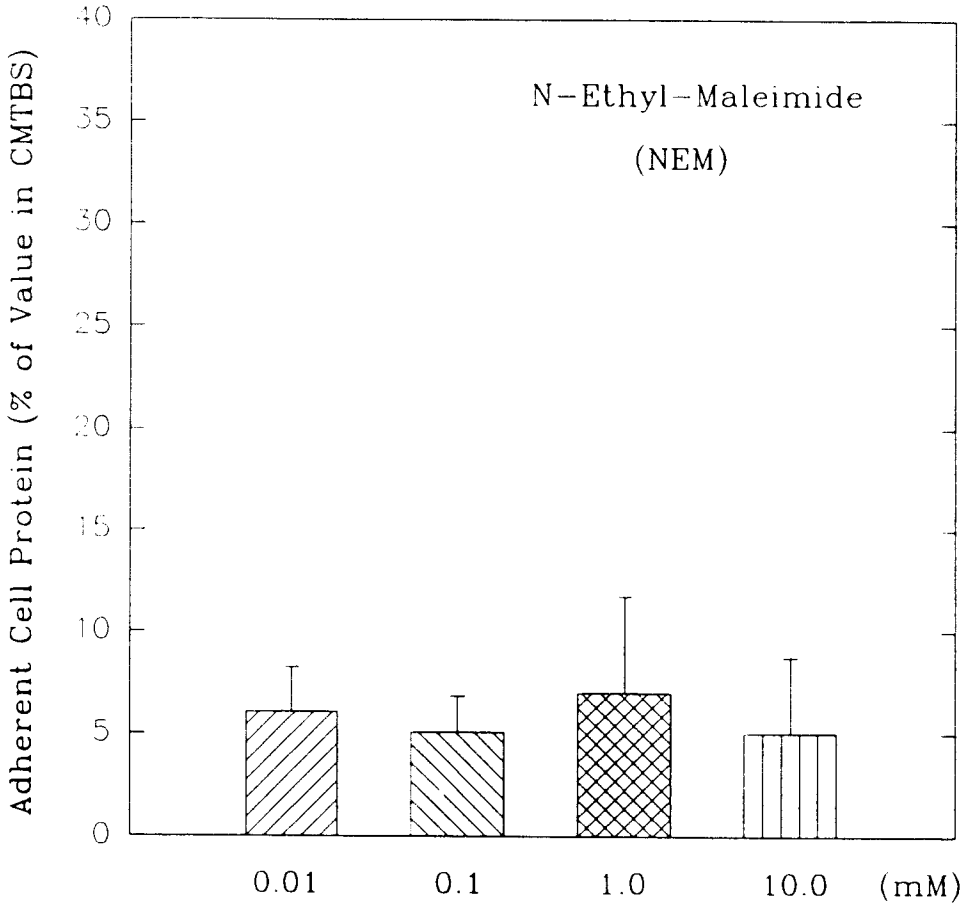


Figure II-9c. Inhibitory effect of NEM on <u>Mytilus</u> hemocyte adhesion <u>in vitro</u>. NEM strongly inhibits hemocyte adhesion. Less than 10 % hemocyte adherence could be detected in 0.01 mM or higher concentrations of NEM ($p<0.05$).

Figure II-9d

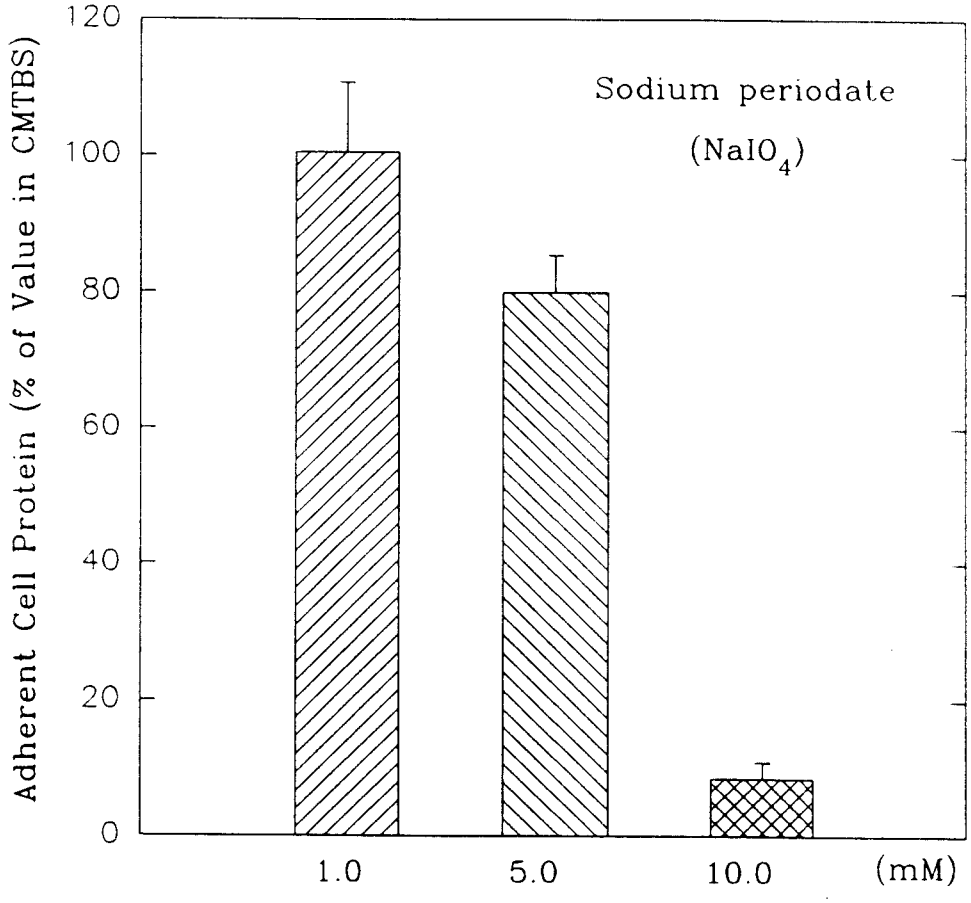


Figure II-9d. Inhibitory effect of $NaIO_4$ on <u>Mytilus</u> hemocyte adhesion <u>in vitro</u>. As in the aggregation test, 10 mM is a critical inhibitory concentration of sodium periodate ($NaIO_4$) on hemocyte adhesion ($p<0.05$). At 5 mM or less, $NaIO_4$ did not block hemocyte adhesion.

Figure II-9e

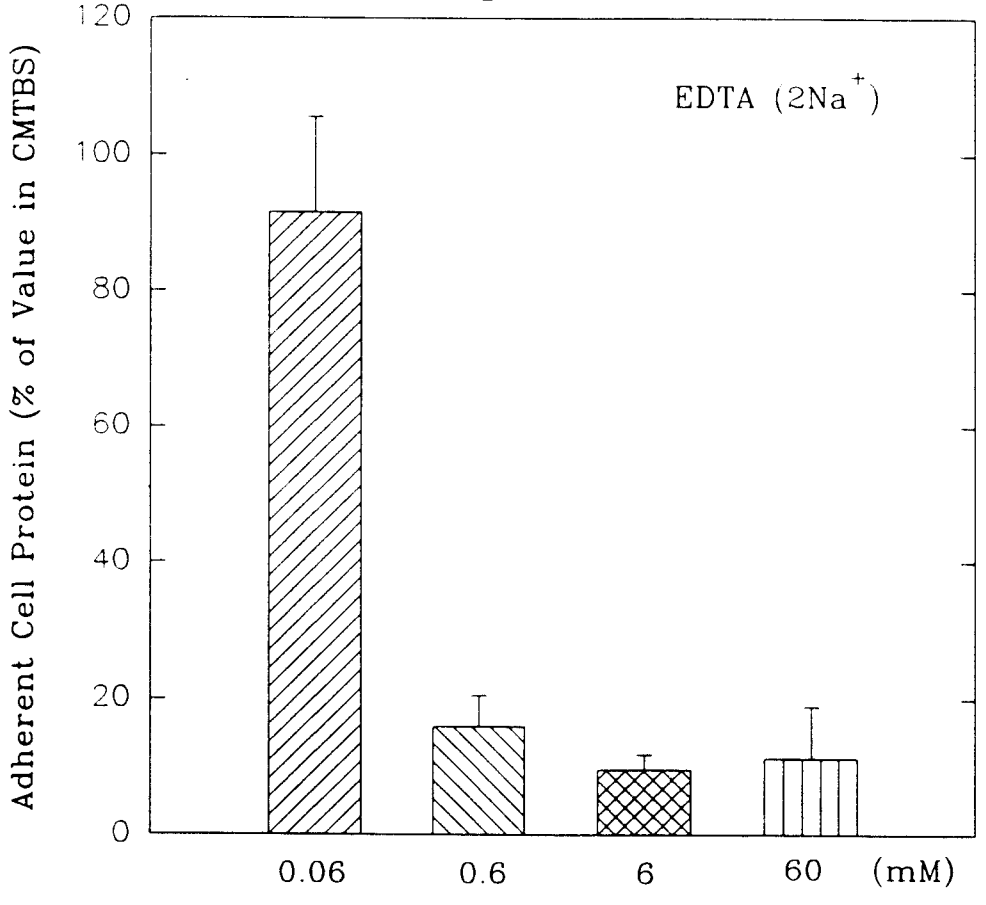


Figure II-9e. Inhibitory effect of EDTA on <u>Mytilus</u> hemocyte adhesion <u>in vitro</u>. At 0.6 mM, EDTA significantly inhibited hemocyte adhesion ($p<0.05$), but this agent was without affect at 0.06 mM.

Figure II-10

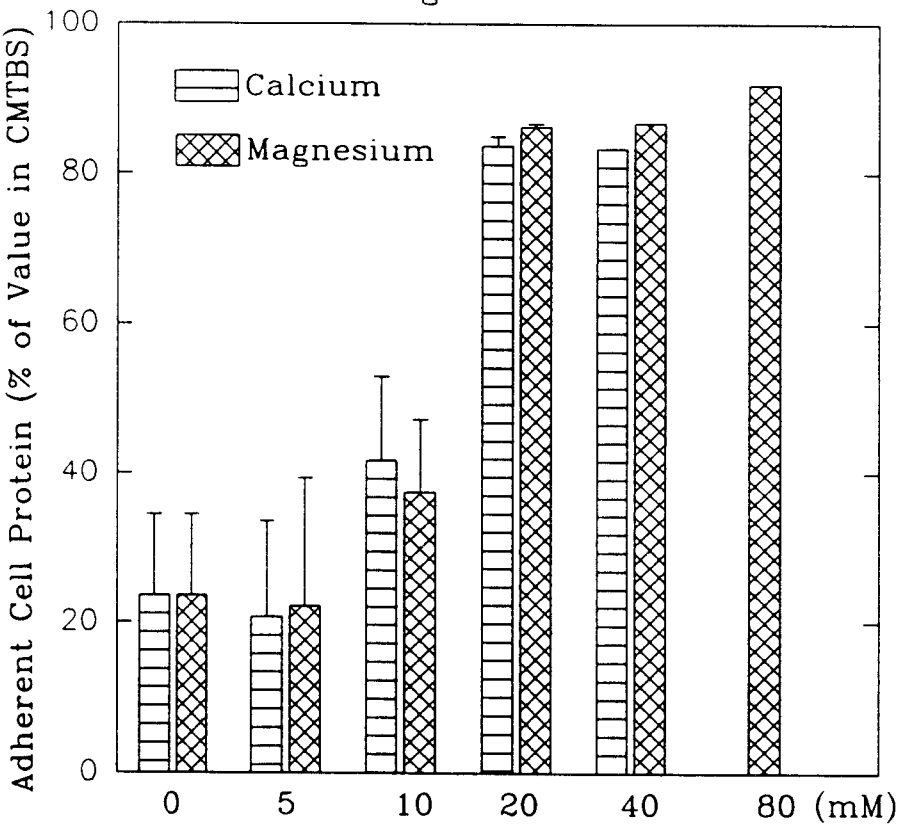


Figure II-10. The effects of calcium and magnesium on <u>Mytilus californianus</u> hemocyte adhesion. After three washes in 30 mM EDTA, hemocytes were suspended in buffered isotonic calcium or magnesium solutions. The cells were loaded into culture plates, then cell adhesion was assayed after 15 min incubation at RT. Cells without EDTA treatment were taken as 100 % adherent. More than 80 % of EDTA-treated hemocytes recovered their adhesion competence at 20 mM or higher concentrations of calcium and/or magnesium isotonic buffer.

CHAPTER III

ADENOSINE AS REGULATOR FOR HEMOCYTE AGGREGATION AND ADHESION IN THE CALIFORNIA MUSSEL (Mytilus californianus).

ABSTRACT

The adhesion of Mytilus hemocytes to plastic and glass substrata was reduced to 30 percent of control values by 10 mM caffeine. When 20 mM adenosine was also present, the inhibitory effect of 10 mM caffeine on hemocyte adhesion disappeared. Similar results were seen when caffeine was added in the presence of adenosine agonists such as 2-chloro-adenosine (2-CLAD), and 5'-N-ethylcarboxamidoadenosine (NECA). In contrast, isobutyl-methyl-xanthine (IBMX) and the adenosine antagonists like cyclo-hexyladenosine (CHA), and R-N^6-phenyl-isopropyladenosine (R-PIA) acted synergistically with 10 mM caffeine to inhibit hemocyte adhesion. In hemocyte aggregation, since 10 mM caffeine was not sufficient to inhibit cohesive aggregation, the ability of adenosine, 2-CLAD, and NECA to overcome 10 mM caffeine was not detectable. However, the combination of IBMX, CHA, or R-PIA with 10 mM caffeine successfully blocked hemocyte cohesive aggregation. No effect was seen in the other two adenosine analogues, 8-phenyl-sulfo-propyltheophylline (8PSPT) and di-deoxyladenosine (DDA), with or without caffeine.

In spread hemocytes, intracellular levels of cAMP were similar to those found in cells treated with 10 mM caffeine. The cAMP levels of hemocytes which were treated with 25 mM caffeine were lower than those found in spread cells. When spread cells were treated with 25 mM caffeine, they rounded up immediately and the intracellular levels of cAMP decreased in one minute. This cAMP level was similar to that found in

cells directly treated with 25 mM caffeine. The trend of change of intracellular levels of cAMP in caffeine with or without adenosine and adenosine analogues was similar to results obtained in adhesion experiments. When hemocytes were treated with 1 mM R-PIA plus 10 mM caffeine, the cAMP levels in hemocytes and hemocyte adhesion were lower than in cells treated with 10 mM caffeine alone. However, both the cAMP level and hemocyte adhesion increased when 20 mM adenosine or 5 mM NECA replaced 1 mM R-PIA.

INTRODUCTION

The cellular aggregation that occurs when mussel hemocytes are traumatized is inhibited by caffeine (Chapter 2). This drug also prevents aggregation of slime mold amoebae (Brenner & Thoms, 1984) and the leukocytes of several invertebrates (Bertheussen & Seljelid, 1978; Ratner & Vinson, 1983; this thesis, Chapter II). However, the mechanism by which caffeine inhibits cellular aggregation and adhesion is not clear. Caffeine is an adenosine analogue, and antagonizes the effect of adenosine on its receptor. It is also a phosphodiesterase inhibitor (Snyder, 1984). These two effects are associated with changes in the intracellular levels of cyclic-5'-adenosine monophosphate (cAMP). In vertebrates, adenosine receptors have been identified as integral membrane proteins which are coupled with adenylate cyclase (reviewed by Fain & Malbon, 1979; Londos & Cooper, 1981; Daly, 1982). As an adenosine receptor antagonist, caffeine can lower intracellular cAMP levels by inactivation of adenylate cyclase conversion of ATP to cAMP. In Dictyostelium, caffeine has been found to be a rapid and reversible inhibitor which affects adenylate cyclase activity via a cAMP-dependent activation system, not via either cAMP receptors or adenosine receptors (Brenner & Thoms, 1984). In contrast as an adenosine receptor

antagonist, caffeine increases intracellular cAMP levels by inhibiting phosphodiesterase (Snyder, 1984). The function of cAMP-specific phosphodiesterase is to catalyze cAMP to AMP, and by this means the intracellular cAMP concentration is normally maintained at a very low level.

Adenosine, which is an essential metabolite, can affect a variety of physiological functions in vertebrates (reviewed by Fain & Malbon, 1979; Daly, 1982). As a modulator of adenylate cyclase, adenosine is either bound to external adenosine receptors (A_1 and A_2), or bound to an internal inhibitory binding site (P-site) to affect adenylate cyclase activity. When adenosine binds to a P-site which is near the catalytic subunit of adenylate cyclase, the enzyme activity is inhibited (Londos & Cooper, 1981). Two subclasses of receptors for adenosine have been identified. A_1 receptors inhibit adenylate cyclase activity to generate cAMP, but A_2 receptors stimulate its function (Daly, 1982). In addition to this functional difference, these two receptors are distinguished by distinct affinities for various adenosine analogues. For example, N^6-substituted adenosine analogues bind A_1 receptors with higher affinity than A_2 receptors. Adenosine receptors of A_1 and A_2 have been identified in a variety of cell types (reviewed by Daly, 1982).

In order to understand whether or not caffeine regulates hemocyte behaviors via adenosine receptors, adenosine and its analogues (adenosine receptor agonists or antagonists) were used together with 10 mM caffeine to reveal potentiation or suppression of the caffeine effect. Also since adenylate cyclase has been identified in various animal cell types to be affected by adenosine or adenosine analogues (Haslam et al., 1978; Londos et al., 1981; reviewed by Daly, 1982), I was curious to know whether or not the intracellular cAMP level in Mytilus hemocytes changed during the process of aggregation and adhesion, and if these concentrations were affected by caffeine. I determined intracellular levels of cAMP after

different treatments. Although mussel hemocyte aggregation and adhesion were suspected to be two distinct processes, it appeared likely that they would be regulated by similar activation pathways, at least in the early stages (Chapter 2). Therefore, in this study estimates of the extent of cell activation are restricted to those revealed by cell adhesion ability.

MATERIALS AND METHODS

Chemicals

Adenosine analogues (2-chloroadenosine, 2-CLAD; 5'-N-ethylcarboxamidoadenosine, NECA; 8-phenyl-sulfo-propyltheophylline, 8PSPT; R-N^6-phenyl-isopropyladenosine, R-PIA) were kindly provided by Dr. T. F. Murray, Pharmacy, Oregon State University. Isobutyl-methyl-xanthine (IBMX), cyclo-hexyladenosine (CHA), di-deoxyladenosine (DDA), di-butyl-cAMP (dbcAMP), cholera toxin, and all other chemicals were purchased from Sigma Co..

2-CLAD, NECA, and 8PSPT have higher binding affinity to A_2 receptors than R-PIA and CHA. But R-PIA and CHA have higher binding affinity to A_1 receptors than 2-CLAD, NECA and 8PSPT. 2-CLAD, NECA, 8PSPT, R-PIA, or CHA cannot bind with P-site. DDA cannot bind with A_1 or A_2 receptors, but can specifically bind with P-site.

Animals

Individual Mytilus californianus larger than 8 cm in length were collected monthly from the rocky intertidal zone at Seal Rock State Park (15 mi south of Newport, OR). On the same day, they were transferred to a filtered, recirculating, continuously aerated sea water system which was maintained at pH 7.6, nitrate < 10 ppm, nitrite < 0.2 ppm, 15°C, and close to normal salinity. Mussels were held in this system for at

least 3 days before being used as a source of hemolymph.

Hemolymph collection

A plastic rod (3 mm diameter) was inserted between the two shells to prevent the shells closing, and sea water was drained from the mantle cavity. Animals were then transferred to a cold room (4°C). Hemolymph was collected from the posterior adductor muscle using a pre-cooled sterile syringe with 18G 1½" needle. With the needle removed, the colorless (slightly opalescent) hemolymph was immediately transferred to pre-cooled sterile tissue culture tubes (Falcon) for further treatment. Hemolymph was collected from each animal once only.

Hemocyte adhesion assay

Mixtures of caffeine and adenosine or adenosine analogues were prepared just before hemolymph was harvested. The volume ratio of caffeine, to adenosine or adenosine analogue, and to hemolymph was 1 : 1 : 2. The final concentration of caffeine was 10 mM or 5 mM. The final concentration of adenosine or adenosine analogues differed, since their solubilities in CMTBS vary. Also caffeine+cholera toxin or caffeine+dbcAMP solution were prepared fresh. After hemolymph had been mixed with test solutions, this was then loaded (50 µl/well) into wells of a pre-cooled (4°C), flat-bottom 96-well tissue culture plate (Corning, New York). Each treatment was loaded in triplicate. After 15 minutes incubation at room temperature, the plasma and unattached cells were removed and each well was washed three times with CMTBS (100 µl/well). As a measure of cell numbers, protein concentrations were determined by means of the BCA protein assay (Pierce). The protocol of protein assay and the relative extent of cell adhesion in different reagents were described in Chapter 2.

Luminescence measurement for intracellular cAMP

Treated hemolymph (1ml) was centrifuged at 20 g for 3 minutes at 4°C to pellet hemocytes which were then immediately frozen in liquid nitrogen. Extractions of the cAMP were performed in 200 µl hypotonic buffer solution (1/10 CMTBS) by sonication at power level 5, tune level 5, for 5 seconds (Contes, Vineland, N.J.).

Intracellular levels of cAMP were estimated using luciferase luminescence. The protocol was that of Ferrero et al. (1984). The method is based on the conversion of cAMP to adenosine 5'-triphosphate (ATP), which was measured by its luminescent reaction with firefly luciferase (Fertel & Weiss, 1978). The rate of ATP generation was measured by a luminometer (1250 LKB-Wallac). To 100 µl of each sample, 5 µl of phosphodiesterase (Boehringer Mannheim, Germany; 0.25 U/mg protein/ml) was added. The mixture was incubated at RT for 5 minutes, and then at 100°C for 1 minute to inactivate any ATP-consuming contaminants. After being cooled in an ice bath, each sample received 100 µl of 10^{-7}M ATP , and 100 µl Breckenridge cycle reagent. The Breckenridge cycle reagent is 50 mM glycylglycine buffer, pH 7.4, which contains 6 mM $MgSO_4$, 100 mM KCl, 0.1 mM phosphoenolpyruvate, 0.5 mg/ml pyruvate kinase, 0.01 mg/ml myokinase. After 7 minutes incubation at RT, the samples were boiled for 3 minutes. Each ice cooled sample (100 µl) was added to 100 µl ATP assay kit solution (Sigma). The rate of luminescence generation was measured by the luminometer for 10 seconds. Data were recorded and printed every second. Concentrations of cAMP were estimated from an ATP standard curve which was obtained each day before the cAMP assay.

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples from different animals. Data are presented as mean ± S.D.. Statistical analysis of data was

performed using paired or unpaired Student's t-test as appropriate. Differences were considered significant when $p<0.05$.

RESULTS

Dose dependence of caffeine inhibition of hemocyte adhesion.

Hemocytes treated with 25 mM caffeine were hardly able to retain their adhesion competence. However, hemocytes retained some adhesion ability in the presence of 10 mM caffeine (Fig. III-1).

The effects of adenosine, inosine, IBMX, 8PSPT and DDA on the inhibitory effect of caffeine on hemocyte adhesion.

At 20 mM, adenosine did not inhibit hemocyte adhesion, but slightly promoted it. Only 20 % of the hemocytes adhered after the hemolymph was treated with 10 mM caffeine. However, when the two drugs were given together (20 mM adenosine and 10 mM caffeine) hemocyte adhesion was significantly enhanced up to 80 % (Fig.III-2). Also, relative to values obtained in 10 mM caffeine, hemocytes adhesion was slightly more in 20 mM inosine + 10 mM caffeine. However, the ability of inosine to overcome inhibition by caffeine was less than that of adenosine (Fig.III-3).

The effect of IBMX was opposite to that of adenosine. It did not inhibit hemocyte adhesion at 0.2 mM, but adhesion values were reduced to 80 % of controls at 2 mM. However, when 2 mM IBMX was combined with 5 mM caffeine, the effects were synergistic; the percentage of cell adhesion decreased to 20 % of controls (Fig.III-4). 8PSPT did not affect hemocyte adhesion (Fig.III-5).

Reversal of caffeine inhibition of hemocyte adhesion.

As in the earlier experiments (above), hemocytes treated

with 10 mM caffeine adhered weakly in 96-well culture plates, approximately 20 % of hemocytes doing so. However, when 2-CLAD was given in the presence of caffeine, hemocyte adhesion increased. More than 60 % of hemocytes adhered in 10 mM caffeine+20 mM 2-CLAD (Fig.III-6). Similarly, adhesion increased in 5 mM NECA/10 mM caffeine to twice that in 10 mM caffeine. Even 1 mM NECA slightly increased adhesion, but not significantly (Fig.III-7). Either 2-CLAD or NECA alone did not change hemocyte adhesion.

The enhancement of caffeine inhibition of hemocyte adhesion.

Neither CHA (0.2 and 2 mM) nor R-PIA (0.1 and 1 mM) when used alone changed mussel hemocyte adhesion capacity. But both these drugs (2 mM CHA and 1 mM R-PIA) significantly enhanced the inhibitory effect of caffeine (10 mM) on hemocyte adhesion (Fig.III-8 & III-9).

The intracellular concentration of cAMP in hemocytes treated with caffeine and/or adenosine analogues.

Concentrations of cAMP in hemocytes held in CMTBS for 1+3 minutes or for 15+3 minutes were not different. When hemocytes were treated with 25 mM caffeine, their cAMP level was 50 % - 65 % of that in CMTBS. When the cells were first treated with CMTBS for 15 minutes and then exposed to 25 mM caffeine for 1 minute, the cAMP concentration was the same as that seen after 15 minutes in 25 mM caffeine (Table III-1). Hemocytes treated with 20 mM adenosine+10 mM caffeine or 5 mM NECA+10 mM caffeine had higher cAMP levels than those treated with 10 mM caffeine alone. However, cAMP levels of samples treated with 1 mM R-PIA+10 mM caffeine were lower than samples treated with 10 mM caffeine alone (Table III-2).

DISCUSSION

Caffeine is the best known inhibitor of Mytilus hemocyte aggregation and adhesion (Chapter 2). In 25 mM caffeine, hemocyte adhesion and aggregation are completely inhibited, but viability is not affected. Similarly, adherent hemocytes round up immediately, and aggregates become loose when they are treated with 25 mM caffeine (data not shown). Effects on both forms of hemocyte behaviors are reversible. When caffeine was removed and replaced with CMTBS, both cell aggregation and adhesion began immediately (data not shown). This suggested that caffeine might have been acting on the cell surface. In addition, hemocytes gradually recovered adhesion competence in 10 mM caffeine. Since caffeine is a stable chemical, its concentration may not decrease in a short period. Therefore, it appears that the loss of inhibitory effect by 10 mM caffeine maybe due to hemocytes decreasing their sensitivity to caffeine. When caffeine-receptor complexes are transported into the cell (internalization), the binding sites on the cell surface are lost, decreasing the cellular sensitivity to caffeine. Such a putative regulatory mechanism resembles the down regulation of specific receptors (Green, 1987).

Among the adenosine receptor agonists, adenosine, 2-CLAD, and NECA have relatively low affinity for A_1 receptors but high affinity for A_2 receptors (Daly, 1982). Results of adenosine+caffeine competition tests on hemocyte adhesion imply that adenosine, 2-CLAD, and NECA can overcome the inhibitory effect of caffeine. CHA and R-PIA which are high rank order agonists of A_1 receptors, however, enhanced caffeine inhibitory activity. This suggests that there are adenosine or adenosine-like receptors on mussel hemocyte surfaces. Although adenosine receptors have been found to be widespread among the classes of chordates (Rozengurt, 1982; Daly, 1982; Siebenaller & Murray, 1986), no adenosine receptor has been so far identified on invertebrate cell surfaces.

Noble (1970) reported that adenosine, AMP, ADP, or ATP at 0.5 mM had no inhibitory effect on sea cucumber coelomocyte aggregation. The results reported here raise the possibility that adenosine receptors may exist in invertebrates.

Like caffeine, IBMX is an antagonist of adenosine receptors, and also is a phosphodiesterase inhibitor. Although the drug alone did not greatly inhibit hemocyte adhesion, it definitely enhanced the caffeine inhibitory effect. Since the slight inhibition seen with DDA (with or without caffeine) was not statistically significant (data not shown), it is suggested that hemocyte activation is not regulated by a "P-site" receptor. Indeed, the adenosine analogues used here (except adenosine and DDA) do not affect "P-site" receptors (Londos & Cooper, 1981; reviewed by Daly, 1982). The negative result obtained with 8PSPT demands an explanation. The nature of 8PSPT makes it difficult for it to pass plasma membranes, and this may restrict the drug to acting at the cell surface, without access to the cytoplasmic "P-site". 8PSPT was hardly soluble in CMTBS, and the concentrations used were lower than 1 mM. Thus, its concentration may have been too low to affect caffeine activity on hemocyte adhesion.

GTP-binding proteins (G protein) are a group of integral membrane proteins with functional domains on the cytoplasmic face. When these proteins bind with guanosine triphosphate (GTP), the activation of the G protein can cause adenylate cyclase to initiate the cAMP signal transduction pathway. Cholera toxin, a protein isolated from the bacterium, Vibrio cholerae, binds to the α subunit of G protein causing continuous adenylate cyclase activation, and then increasing intracellular cAMP level (Gilman, 1984; Neer & Clapham, 1988). This toxin in the presence or absence of caffeine did not affect hemocyte adhesion (data not shown). Since it is known that catecholamines, prostaglandin E_2, histamine, or ACTH can affect adenylate cyclase activity (Jakobs et al., 1981; Kather & Simon, 1981), their abilities to change hemocyte behavior

were examined in the presence or absence of caffeine. No effects were seen (data not shown). The hemocytes treated with cAMP, and dbcAMP behaved normally, and these two drugs did not overcome the inhibitory effect of caffeine (data not shown). Since there is no evidence for G proteins, cAMP receptors, or receptors for the tested hormones on mussel hemocyte surfaces, more studies are needed to reveal the basis for the negative results.

Since truly naive mussel hemocyte could not really be obtained in vitro, the baseline cAMP level in resting cells could not be measured. Since cAMP levels in cells held in 25 mM caffeine treatment for 15 minutes or in CMTBS+caffeine were closely similar, and since cell morphology resembled the un-activated condition (Chapter 2), the cAMP level in 25 mM caffeine treatment is our best indication of values in naive cells. However, it could also be that the cAMP level of hemocytes in CMTBS reached a plateau in 4 minutes and remained here for at least 18 minutes. A rapid change of cAMP level during cell activation has been reported (reviewed by Firtel et al., 1989). The cGMP level in Dictyostelium reaches its peak within 10 seconds after activation. Due to the difficulty of obtaining resting mussel hemocytes for determination of cAMP levels, more study is needed to assess how fast the hemocyte cAMP level can change. As seen in Table III-2, R-PIA synergized with suboptimal concentrations of caffeine to inhibit hemocyte adhesion. It also lowered the levels of cAMP in hemocytes when it was present with caffeine. Although the increases with adenosine or NECA treatments varied between different experiments, they were consistent. The variation may have reflected the hemolymph quality. If the hemolymph was collected free of contamination by other tissues, the hemocyte condition could be regarded as close to the resting stage. However, this was rarely achieved. Almost invariably, despite efforts to avoid this, one experiences some contamination of mucus, tissue debris, or gametes.

Based on these results, it appears that the inhibitory effect of caffeine on hemocyte activation, at least as measured by hemocyte adhesion, is mediated via adenosine receptors, and that these receptors cause adenylate cyclase to alter the level of intracellular cAMP. Since caffeine is an antagonist of A_1 and A_2 receptors and has similar affinity constants for both (reviewed by Daly, 1982), the mechanism by which caffeine inhibits hemocyte activation may depend on occupation of all the adenosine receptors on the cell surface. In such a case neither stimulation nor inhibition of adenylate cyclase would occur. Such cells would be maintained in a state close to the naive state. At 10 mM, caffeine keeps the hemocytes in a state of incipient activation. When adenosine or its analogues were added with 10 mM caffeine, the transient balance of their incipient state was interrupted. Adenosine, 2-CLAD, and NECA which stimulate adenylate cyclase activity via A_2 receptors, activated the cells by increasing intracellular cAMP levels. In contrast, the inhibitory effects of CHA and R-PIA which act on A_1 receptors would be due to decreased cAMP levels inside the treated cells.

Figure III-1

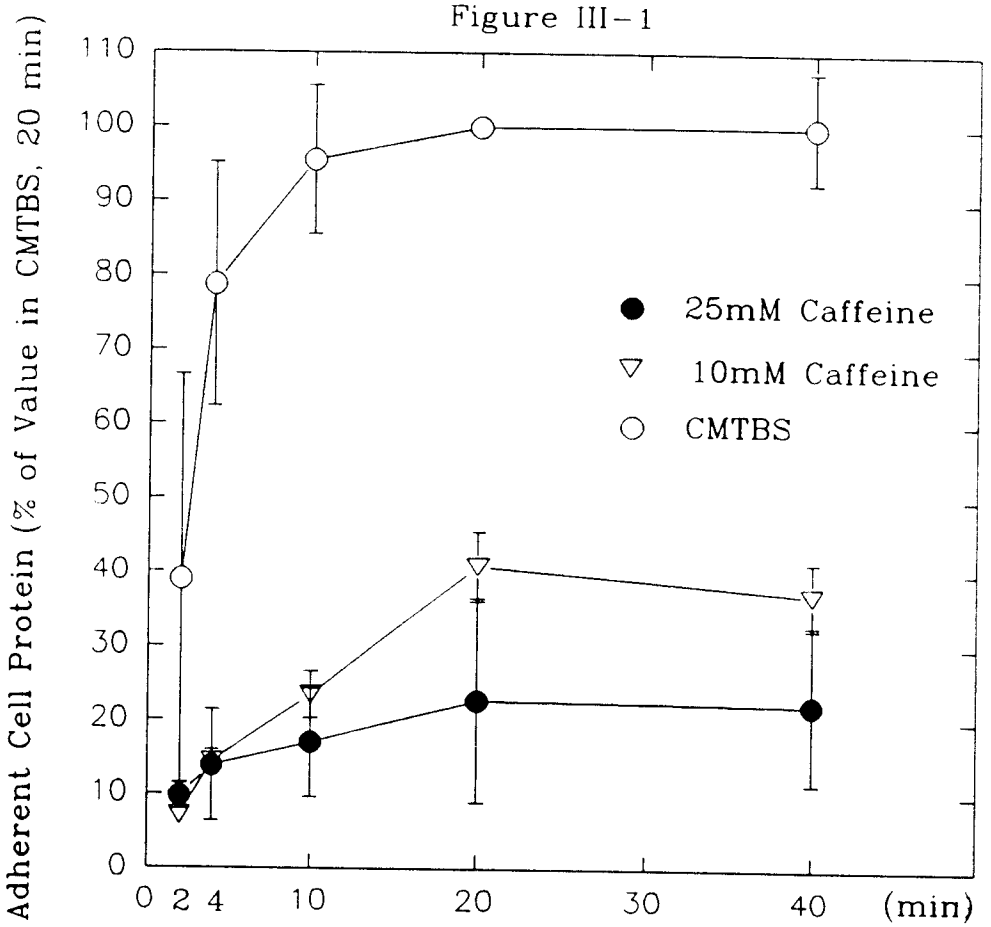


Figure III-1. The influence of caffeine on adhesion of <u>Mytilus californianus</u> hemocytes to a plastic substratum. Hemocytes in 10 mM caffeine were incompletely inhibited, whereas inhibition was complete in 25 mM caffeine. The percentage of cell adhesion in 10 mM caffeine obviously increased after 20 minutes incubation ($p<0.05$). CMTBS = calcium and magnesium-containing tris-buffered saline. Caffeine was dissolved in the same buffer.

Figure III-2

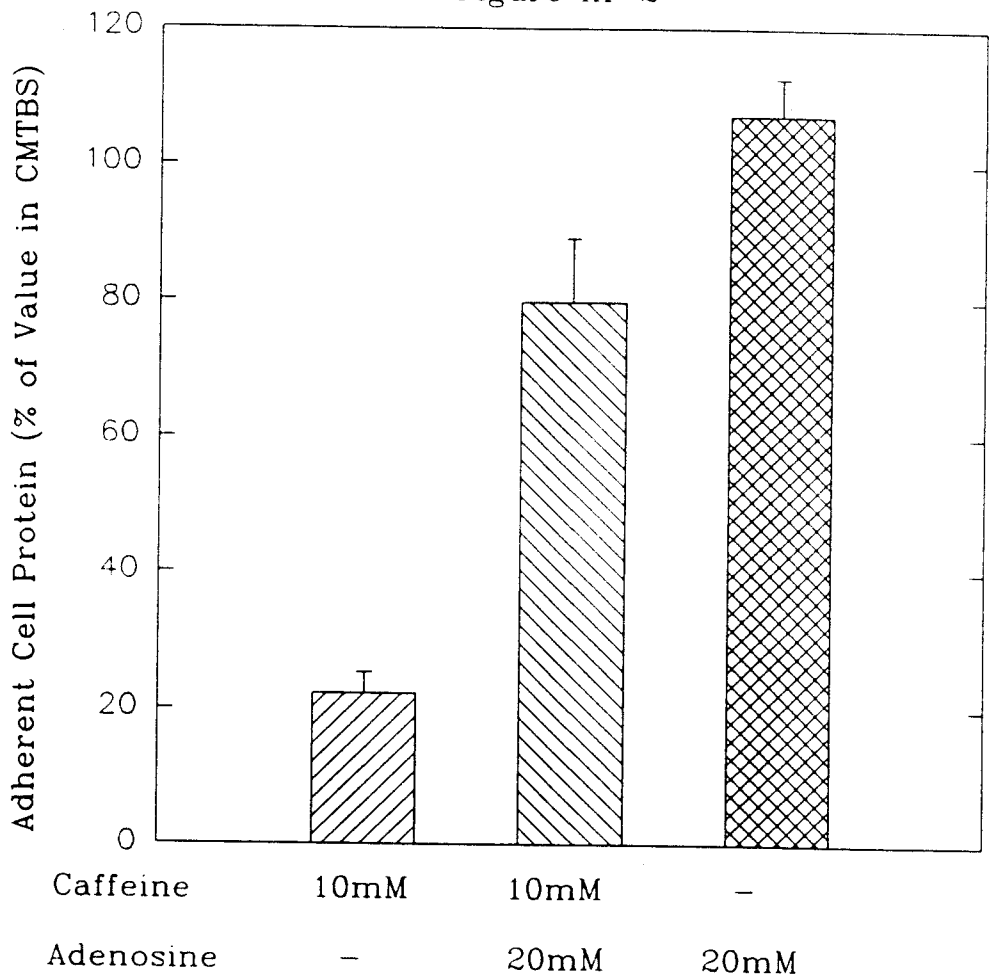


Figure III-2. The influence of adenosine and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Adenosine (20 mM) significantly overcame the inhibitory effect of 10 mM caffeine ($p<0.05$).

Figure III-3

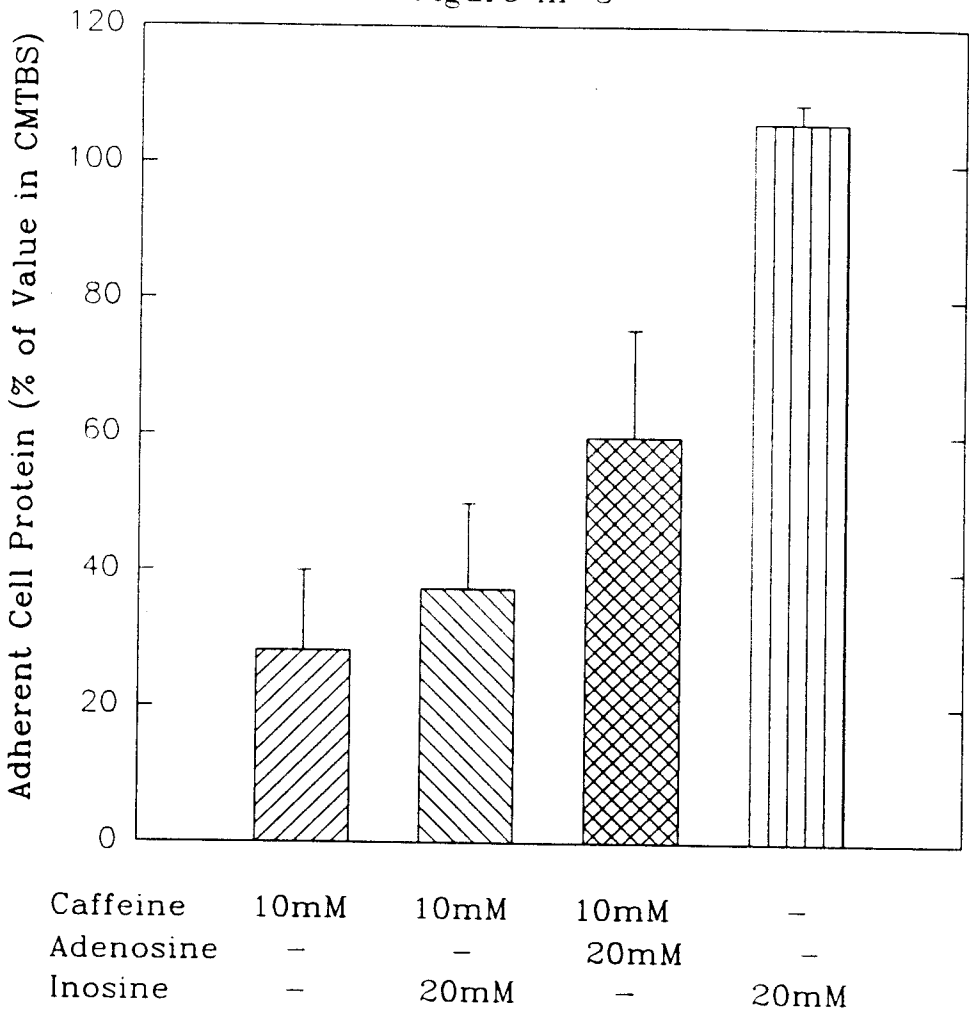


Figure III-3. The influence of inosine, adenosine and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Hemocyte adhesion in 20 mM inosine+10 mM caffeine was higher than in 10 mM caffeine (paired t-test, $p<0.05$). In comparison with 20 mM adenosine, 20 mM inosine was less efficient at interfering with the inhibitory effect of 10 mM caffeine (paired t-test, $p<0.05$).

Figure III-4

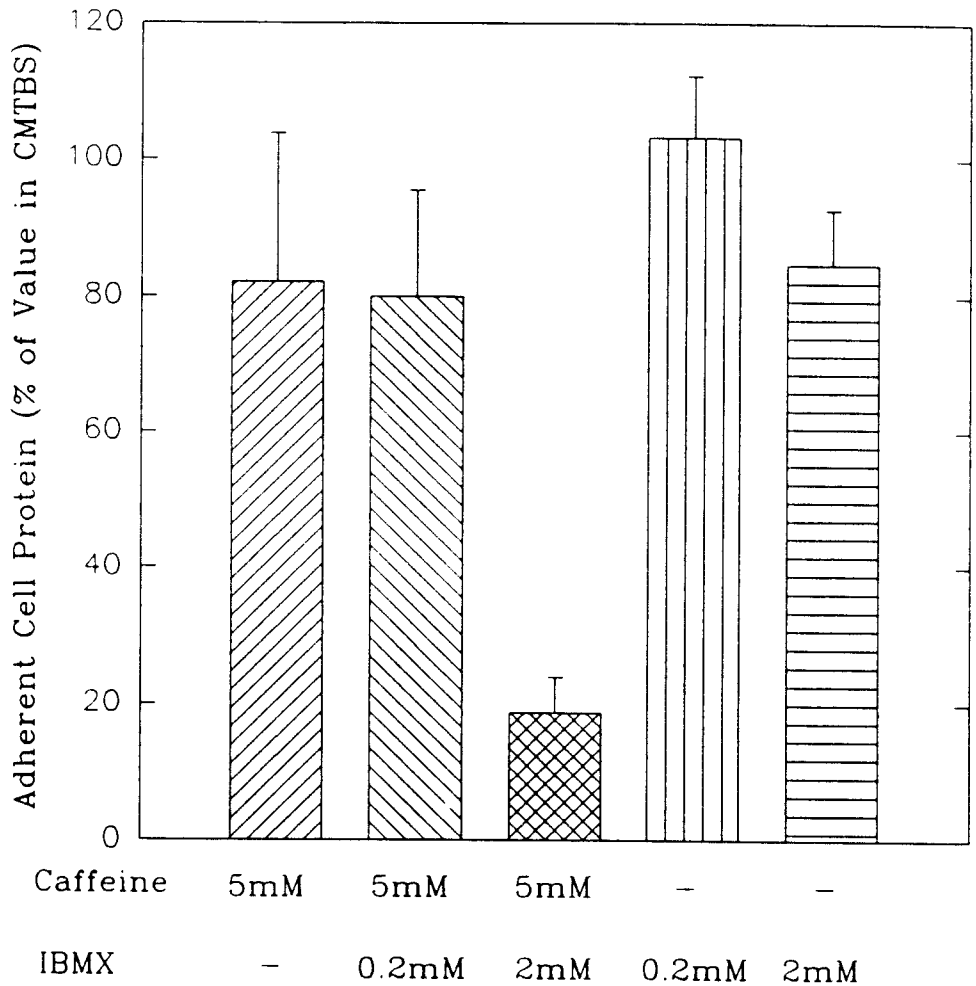


Figure III-4. The influence of IBMX and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. At 2 mM, IBMX slightly reduced hemocyte adhesion ($p<0.05$). Like inhibition by 5 mM caffeine, the inhibition of hemocyte adhesion by 5 mM caffeine+0.2 mM IBMX was not significant. However, at 2 mM, IBMX synergized with caffeine to significantly inhibit hemocyte adhesion ($p<0.05$).

Figure III-5

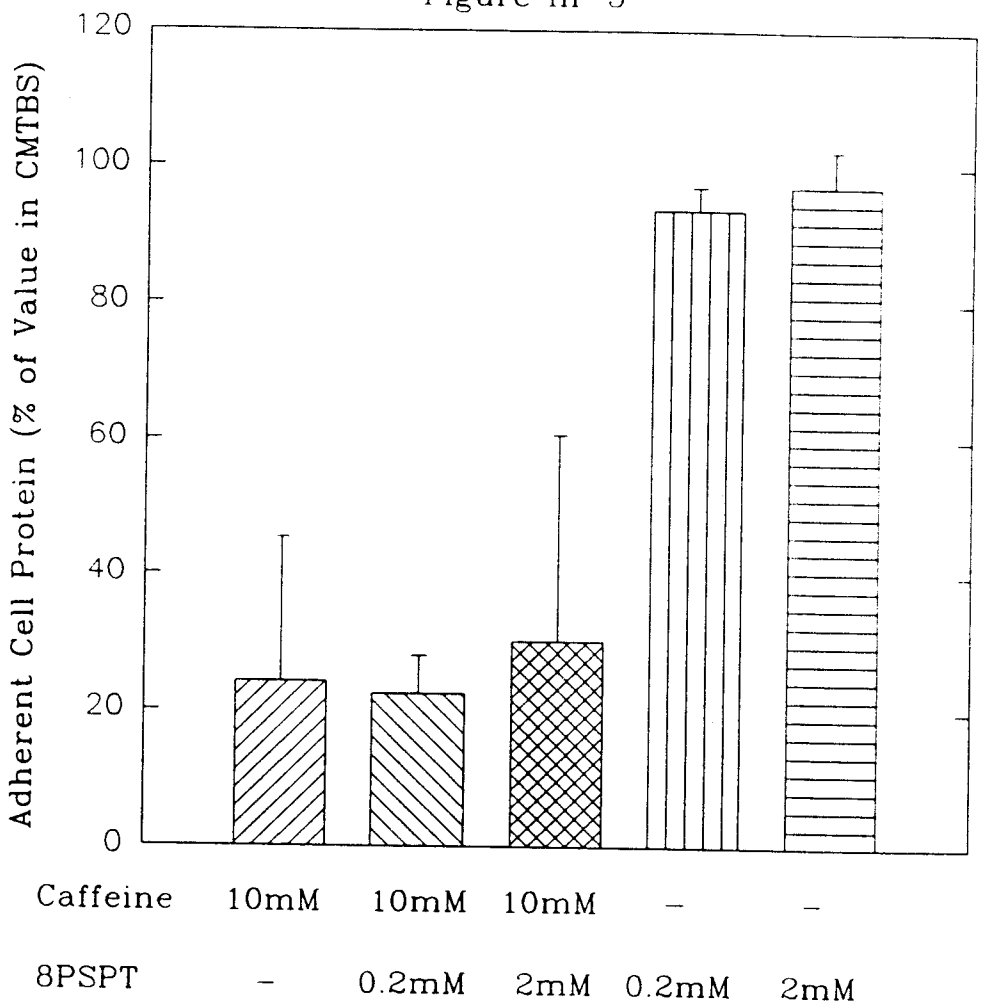


Figure III-5. The influence of 8PSPT and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. No marked effect of 8PSPT was found on hemocyte adhesion with or without caffeine present.

Figure III-6

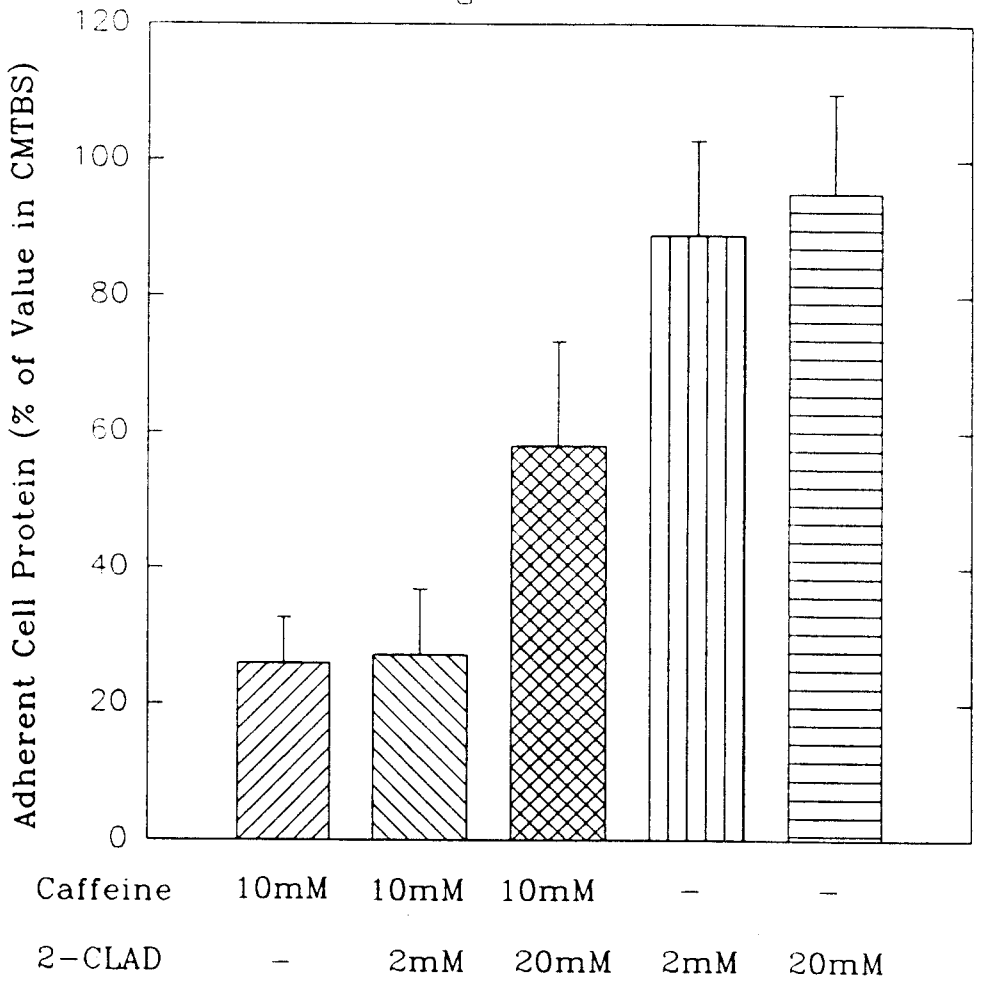


Figure III-6. The influence of 2-CLAD and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Neither 2 mM nor 20 mM 2-CLAD affected hemocyte adhesion. Hemocyte adhesion in 2 mM 2-CLAD+10 mM caffeine was stronger than in 10 mM caffeine alone (paired t-test, $p<0.05$). And 60 % of hemocytes retained their adherent capacity in 10 mM caffeine+20 mM 2-CLAD, the value being significantly higher than that in 10 mM caffeine ($p<0.05$).

Figure III-7

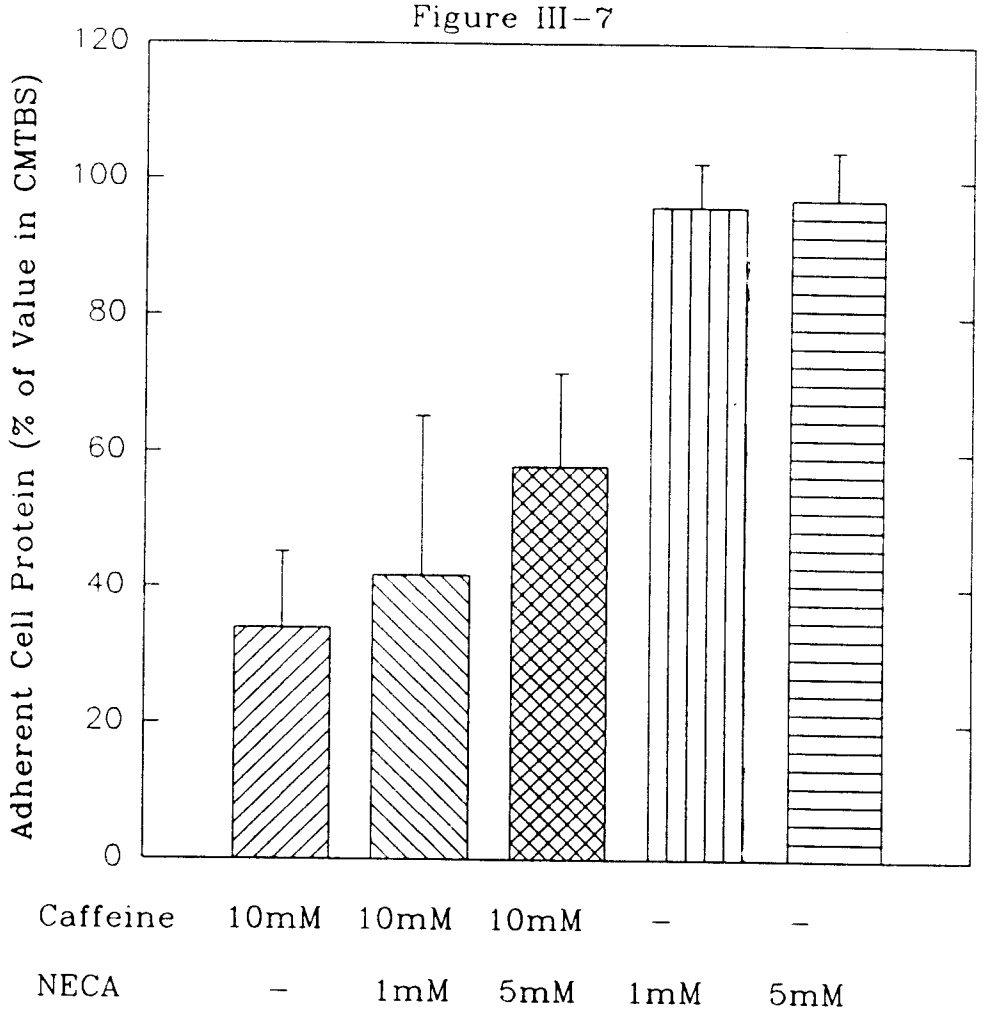


Figure III-7. The influence of NECA and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Hemocyte adhesion was not affected in 1mM or 5 mM NECA alone. In comparison with hemocyte adhesion in 10 mM caffeine, significantly more cells adhered in 10 mM caffeine+5 mM NECA ($p<0.05$). Hemocyte adhesion in 10 mM caffeine was not enhanced by 1 mM NECA.

Figure III-8

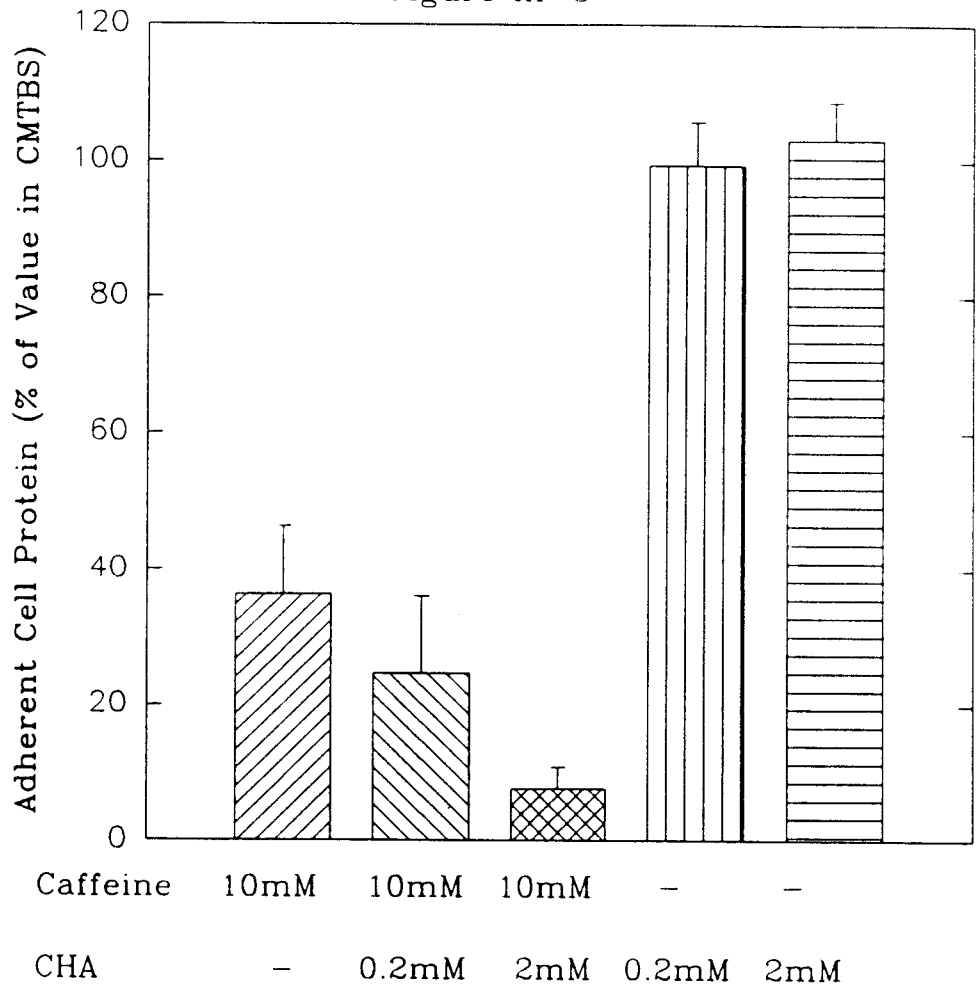


Figure III-8. The influence of CHA and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Hemocyte adhesion did not change in either 0.2 mM or 2 mM CHA. The mixture of 10 mM caffeine+0.2 mM CHA slightly decreased hemocyte adhesion, but this was not statistically significant. Hemocyte adhesion was significantly decreased in 10 mM caffeine+2 mM CHA ($p<0.05$).

Figure III-9

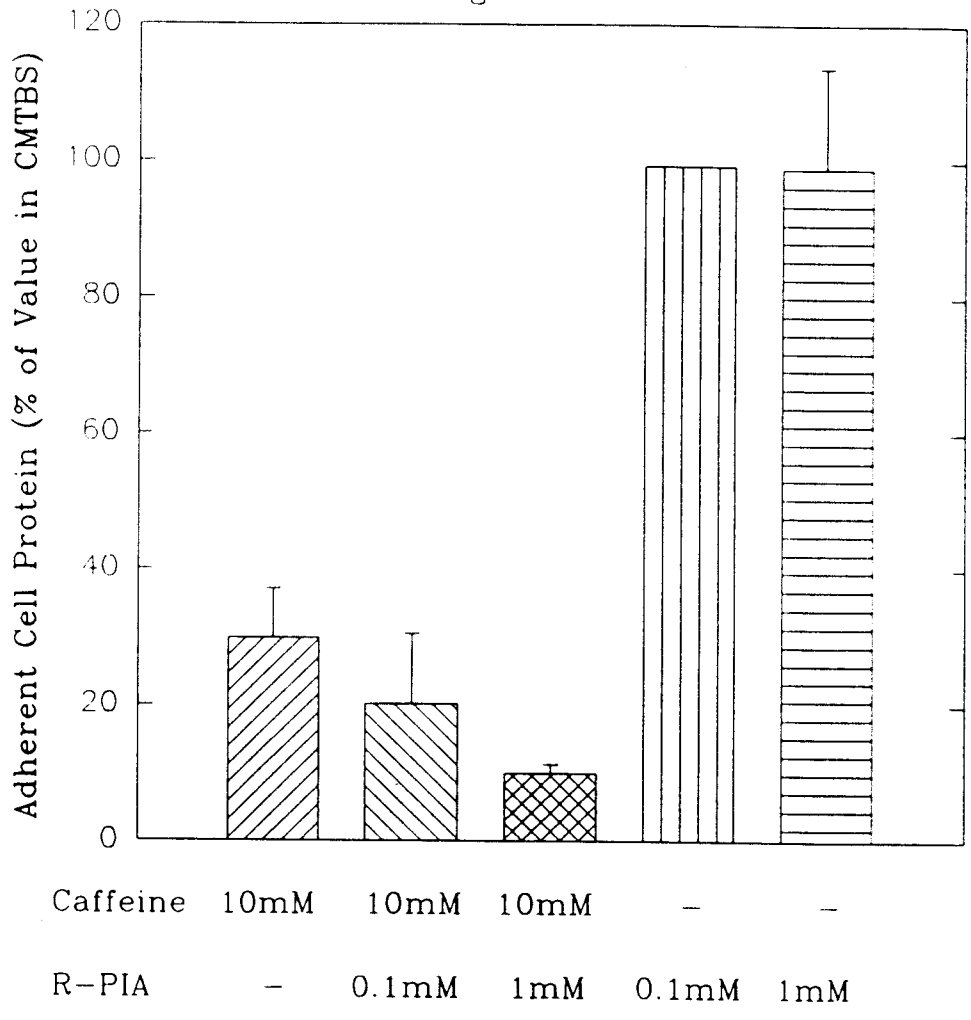


Figure III-9. The influence of R-PIA and/or caffeine on <u>Mytilus californianus</u> hemocyte adhesion. Hemocyte adhesion did not change in either 0.1 mM or 1 mM R-PIA. Hemocyte adhesion was significant decreased in 10 mM caffeine+1 mM R-PIA ($p<0.05$). The mixture of 10 mM caffeine+0.1 mM R-PIA slightly, but not significantly, decreased hemocyte adhesion.

Table III-1. The intracellular levels of cAMP in *Mytilus californianus* hemocytes following exposure to CMTBS or caffeine.

	cAMP Concentration		
Treatment	Exp. 1	Exp. 2	Exp. 3
1. CMTBS (1+3)	5.8 μM	-	2.5 μM
2. CMTBS (15+3)	5.6 μM	6.5 μM	2.25 μM
3. 25 mM caffeine	3.65 μM	3.1 μM	1.65 μM
4. CMTBS-caffeine	3.55 μM	-	1.5 μM

Treatment 1: the process of hemolymph collection and aliquoting was completed in less than 1 minute at 4°C. The cells were then spun down at 20 g for 3 minutes. The 1+3 means that the cells were frozen in liquid nitrogen within 4 minutes of removal from the mussel.

Treatment 2: after being mixed with CMTBS, the hemolymphs were incubated at RT for 15 minutes.

Treatment 3: hemocytes were incubated at RT for 15 minutes in 25 mM caffeine.

Treatment 4: Cells were treated with CMTBS for 15 minutes, and then exposed to 25 mM caffeine for 1 minute.

* Because the original cell number varies between each hemolymph collection, the data presented here cannot be pooled together.

Table III-2. The intracellular levels of cAMP in Mytilus californianus hemocytes exposed to caffeine with or without adenosine analogues.

	cAMP Concentration		
Treatment*	Exp. 1	Exp. 2	Exp. 3
10 mM caffeine	3.85 μM	6.5 μM	2.45 μM
10 mM caffeine/ 20 mM adenosine	9.0 μM	-	3.15 μM
10 mM caffeine/ 1 mM R-PIA	1.9 μM	2.5 μM	-
10 mM caffeine/ 5 mM NECA	6.1 μM	6.8 μM	-
CMTBS	-	8.0 μM	5.9 μM

* After being mixed with CMTBS or appropriate reagents, the treated hemolymph were incubated at RT for 15 minutes. The hemocytes were then spun down at 20 g for 3 minutes. The cell pellet was stored in liquid nitrogen immediately.

CHAPTER IV

THE POSSIBLE ROLE OF PROTEIN KINASE C IN HEMOCYTE AGGREGATION AND ADHESION IN THE CALIFORNIA MUSSEL (*Mytilus californianus*).

ABSTRACT

The change from an unsticky to a sticky state that occurs when mussel hemocytes are collected has been shown previously to be inhibited by caffeine. This inhibition can be overcome by the addition of phorbol ester (PMA), calcium ionophore (A23187), or calcium ions. Since phorbol ester can mimic the diacylglycerol (DAG) effect of activating protein kinase C (PKC), and diacylglycerol comes from phospholipid, it was inferred that an endogenous signal via a phospholipid transduction pathway may lead to hemocyte aggregation and adhesion. Verapamil, a calcium channel blocker, also inhibits hemocyte aggregation and adhesion. Since PKC is a calcium-dependent protein kinase, it appears likely that phospholipid/Ca^{++} turnover may serve as an intracellular message in hemocytes to activate PKC which then regulates hemocyte aggregation and adhesion. This is supported by the further observation that a protein kinase C inhibitor, H7, interfered with hemocyte aggregation and adhesion. PMA overcame the inhibitory effect of sodium periodate ($NaIO_4$), but not EDTA or N-ethylmaleimide (NEM). Thus, the ability of $NaIO_4$ to inhibit hemocyte adhesion may be due to its effect on a receptor, whereas EDTA or NEM, in contrast, may affect adhesion molecules.

INTRODUCTION

Cells alter their behavior in response to changes in their environments. When cells detect differences such as altered concentrations of specific molecules, they respond appropriately. Basically, cell surface receptors recognize and bind specific signal molecules. Since these are present in the extracellular environment, and since, for most of them, the plasma membrane serves as a barrier against entry, cells need transduction systems that precisely translate external information signals to intracellular signals. On binding their ligands, receptors normally change the conformation of their cytoplasmic domain (Kasuga et al., 1982; Springer, 1990; Ullrich & Schlessinger, 1990). This alteration initiates specific enzyme cascade reactions leading to changes in the intracellular concentrations of molecules such as Ca^{++}, cAMP, or cGMP (Takai et al., 1982; Dixon et al., 1987; Neer & Calpham, 1988). These molecules now are recognized as intracellular signal molecules. They can induce specific protein kinases to phosphorylate their target proteins, thus regulating cell behaviors (Kasuga et al., 1981; Heyworth et al., 1989; Jiang et al., 1990). Two pathways of intracellular signal transduction have been particularly well studied: the cAMP pathway (Rodbell et al., 1981) and the phospholipid/Ca^{++} pathway (Nishizuka, 1984). Both of these occur in a variety of cell types.

The calcium- and phospholipid-dependent protein kinase was discovered by Nishizuka and his colleagues in 1977. Since this enzyme is the only protein kinase known to require calcium for activation, it was named protein kinase C (PKC). After binding its ligand, a specific receptor such as the acetylcholine receptor regulates a G protein to activate phospholipase C (Neer & Calpham, 1988). The inositol phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), is then degraded to 1,2-diacylglycerol (DAG) and inositol 1,4,5-

triphosphate (IP_3) by activated phospholipase C. These two products have separate effects on the phospholipid/Ca^{++} pathway. IP_3 can induce calcium ions to be released from calcium storage organelles (such as mitochondria and calcisomes) to activate protein kinase C. DAG is a protein kinase C activator which has a greater potential than calcium to activate PKC (reviewed by Nishizuka, 1986). Actually DAG appears able to lower the requirement of PKC for calcium (Takai et al., 1982). Phospholipid and PKC have been found to be involved with numerous cell activation processes including tumor promotion (Nishizuka, 1984), cell growth (Vicentini & Villereal, 1986), cell secretion (Chakravarty et al., 1990), cell adhesion (Rothlein & Springer, 1986; Juliano, 1989), platelet aggregation, the respiratory burst (Mege et al., 1989), and the immune response (Melloni et al., 1986; Desai et al., 1990; Evans et al., 1990; Myers et al., 1990).

As defined in this study, activation of mussel hemocytes is manifested as increasing hemocyte aggregation and adhesion. Mussel hemocyte activation is rapid and occurs spontaneously when hemolymph is collected. In mussel hemocyte activation studies, caffeine serves as a reversible inhibitor. Its inhibitory effect can be overcome by adenosine and its agonists. The possible involvement of adenosine receptors and a cAMP signaling pathway in hemocyte activation have been discussed (Chapter 3). The other signal transduction pathway, phospholipid/Ca^{++}, was believed to be involved in hemocyte activation also. In order to determine whether PKC is involved in mussel hemocyte aggregation and adhesion, we used PMA and A23187 to activate caffeine-inhibited hemocytes in this study.

MATERIALS AND METHODS

Reagents

Phorbol ester (PMA), calcium ionophore (A23187), H7 (1-

(5-isoquinolinylsulfonyl)-2-methylpiperazine), verapamil, and EGTA were purchased from Sigma Co., (St. Louis, MO). PMA stock solution was prepared by making 2 mg/ml in 100 % DMSO. The calcium ionophore A23187 (10 mg) was dissolved in 10 % DMSO to give a 1 mM stock solution. H7 stock solution (100 mM) was prepared by dissolving the drug in 2 % DMSO. Each stock solution was stored as 50 µl aliquots at -70°C. Test concentrations were prepared by dilution in CMTBS. DMSO at 0.5 % or less has no effect on mussel hemocyte viability and morphology. EGTA was dissolved in CMFTBS (calcium and magnesium free Tris-buffered saline) and was neutralized to pH 7.4 by NaOH. Verapamil is a light-sensitive chemical which was dissolved in CMTBS and stored in the dark.

Hemocyte aggregation assay

The protocol for the cell aggregation assay including hemolymph collection, hemolymph and reagent mixing, and slide gyration were the same as in Chapter 2. The extent of cell aggregation was distinguished and recorded: no aggregation (0°), weak aggregation (1°), and cohesive aggregation (2°).

Hemocyte adhesion assay

The whole protocol for measuring cell adhesion including the treatment of hemolymph, and other steps, were the same as in Chapter 2. The extent of cell adhesion in different reagents is relative to those in CMTBS. The values for cell adhesion in CMTBS were taken as 100 %.

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples from different animals. Data are presented as mean ± S.D.. Statistical analysis of data was performed using paired or unpaired Student's t-test as appropriate. Differences were considered significant when $p<0.05$.

RESULTS

The effect of calcium on hemocyte aggregation and adhesion.

In 15 mM EGTA (pH 7.4), mussel hemocyte aggregation (Fig.IV-1a) was significantly inhibited ($p<0.05$), but cell adhesion (Fig.IV-1b) was not affected. Neither hemocyte behavior was much affected in 7.5 mM EGTA.

Relative to the low hemocyte adhesion seen in 10 mM caffeine, high adhesion competence was seen in 10 mM caffeine with 50 mM or 100 mM Ca^{++}, almost the same as in CMTBS. Such an effect did not occur in caffeine+magnesium solution, even when the magnesium concentrations were as high as 100 mM or 200 mM (Fig.IV-2).

At 1 mM, verapamil significantly inhibited hemocyte aggregation and adhesion ($p<0.05$, Table VI-1), although the value in experiment 2 was high. At 0.1 mM, verapamil was not inhibitory.

The effect of PMA+caffeine or A23187+caffeine on hemocyte aggregation.

When the hemolymph was treated with 25 mM caffeine, more than 90 % of hemocytes remained free after 100 rpm gyration at RT for 15 minutes. With PMA also present (Fig.IV-3), the aggregation competence of hemocytes was maintained. Less than 20 % of hemocytes remained free when the 25 mM caffeine solution contained 0.01 μg/ml PMA. Most cells (>90 %) aggregated in 25 mM caffeine with 0.1, 1, or 10 μg/ml PMA. Similar results were obtained when the cells were treated with A23187+caffeine (Fig.IV-4). In 0.01 μM A23187+25 mM caffeine, hemocytes did not adhere well, as seen in 25 mM caffeine. However, in concentrations of A23187 equal to or greater than 0.1 μM, individual hemocytes spread on the substratum and increased their adhesiveness. In order to reveal any possible activation effect due to DMSO, this was added to caffeine solutions at the same concentrations that were in the PMA or

A23187 solutions. At 0.5 % and 0.125 %, no significant effects were seen.

The ability of PMA to overcome the inhibitory effects of caffeine, EDTA, NEM, or $NaIO_4$ on hemocyte adhesion.

As in the aggregation assay, the hemocytes retained almost all of their adhesion capacity when 10 μg/ml PMA was present in 25 mM caffeine (Fig.IV-5). Compared to hemocyte adhesion in 10 mM $NaIO_4$, hemocyte adhesion in 10 mM $NaIO_4$ + 10μg/ml PMA increased about 4 times. But 10 μg/ml PMA did not enhance hemocyte adhesion in 0.2 mM NEM or 60 mM EDTA solutions.

The effect of H7 on hemocyte aggregation and adhesion.

In hemolymph treated with 10 mM H7, hemocytes lost 50 % of their aggregation competence. This interference did not occur in 1 mM or 0.1 mM H7 solutions (Fig.IV-6a). The inhibitory effect of H7 on hemocyte adhesion (Fig.IV-6b) was similar to the effect on aggregation. The concentration of H7 at which interference with cell adhesion was evident was 10 mM. This was quite precise, because 1 mM or 0.1 mM did not have any inhibitory effects (Fig.IV-6b).

DISCUSSION

Among invertebrates, calcium ion has been reported to serve as an intracellular message in sponges (Dunham et al., 1983), and in molluscs (Shozawa & Suto, 1990). In addition, phosphoinositide and protein kinase C have also been demonstrated to be involved in sponge cell aggregation (Müller et al., 1987; Gramzow et al., 1988). The results reported here further corroborate the observation that protein kinase C not only exists in invertebrate cells, but also regulates metabolic processes by the same control system as seen in

higher animals.

The inhibitory effect of EDTA on hemocyte aggregation and adhesion was reported in Chapter 2. EGTA (ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid), a calcium-specific chelator, was used in this study. Since the normal calcium concentration in mussel hemolymph is about 10 mM (Bayne et al., 1976), two concentrations of EGTA, 15 mM, and 7.5 mM were selected. At 15 mM, EGTA almost completely blocked hemocyte aggregation, but was only partially inhibitory towards hemocyte adhesion. However, in 7.5 mM EGTA, neither hemocyte behavior changed significantly. These results, combined with EDTA data in Chapter 2, suggested that mussel hemocyte adhesion is divalent cation-dependent, and hemocyte aggregation is Ca^{++}-dependent. However, for two reasons, the inhibitory effects of EDTA and EGTA should not be so simply interpreted. First, as discussed in Chapter 2, hemocyte adhesion was inhibited in 6 mM EDTA which chelated only a portion of the divalent cations in mussel hemolymph. Second, although 7.5 mM EGTA did not inhibit hemocyte adhesion, it could retard hemocyte spreading (data not shown). It appears that hemocyte adhesion is divalent cation-dependent and hemocyte spreading is a calcium concentration-sensitive process. But the possibilities exist that EDTA may serve not only as a chelator (Kenney et al, 1972; Kanungo, 1982), and the divalent cations in hemolymph may play roles other than in hemocyte activation, for example serving as cofactors of adhesion molecules.

In order to reveal the possible role of extracellular Ca^{++} in hemocyte activation, two experimental approaches were used. Verapamil, a calcium channel blocker, reduced hemocyte aggregation and adhesion (Table IV-1). The single high adhesion value seen in experiment 2 (75.3%) may have been due to inappropriate sample preparation. That is, cells may have been partially activated before they were exposed to verapamil. The second approach was to test the hemocyte

adhesion in calcium+caffeine solution. Ca^{++} concentrations >50 mM significantly blocked the inhibitory effect of caffeine. This did not occur in magnesium supplements as high as 100 mM or 200 mM. Based on these results, it is suggested that there are calcium channels located on mussel hemocyte surfaces, which may be gated by external calcium concentration. The concentration of Ca^{++} in mussel hemolymph is similar to that in sea water, about 10 mM (Bayne et al., 1976). This is more than ten thousand times higher than the generally found intracellular level. Therefore, mussels must have efficient means by which to maintain very low intracellular levels of calcium in resting hemocytes. However, the regulatory mechanisms seem to break down at 50 mM or higher concentration of extracellular Ca^{++}. Such high calcium concentrations may occur transiently around activated cells or in wounded tissues, activating hemocytes in the vicinity.

A23187 is a Ca^{++}-specific ion channel (Evans et al., 1990). When it integrates into the plasma membrane, calcium ions enter and intracellular calcium concentrations increase immediately. In the hemocyte aggregation assay, hemocytes retained their aggregation capacity in A23187+caffeine. This further implies a role for external calcium ions in hemocyte activation. However, to verify the involvement of protein kinase C in hemocyte activation, more research is required. For example, protein kinase C (PKC) is not the only protein which can be activated by calcium: phosphodiesterase does not become active until one of its subunits, calmodulin, binds with calcium ions to change its molecular conformation.

In this study, PMA has also been found to promote hemocyte aggregation and adhesion in concentrations of caffeine that are normally inhibitory. PMA can mimic the activity of DAG, an endogenous PKC activator, to specifically activate protein kinase C, and it has been used in this way to study the results of PKC activation in a variety of cell types. In light of this, our results imply that the activity

of protein kinase C in mussel hemocytes is mediated by calcium ion concentration and phospholipid during aggregation and adhesion. In addition, the inhibitory effects of H7, a protein kinase inhibitor, also support the possibility of a central role for protein kinase C in hemocyte activation.

In the sponge, Geodia cydonium, cell aggregation has been extensively studied (reviewed by Müller, 1982; Müller et al., 1987; Gramzow et al., 1988). Two aggregation-related molecules, aggregation factor (AF) and aggregation receptor (AR), have been isolated and characterized (Müller, 1982). When AF binds to AR, the phosphoinositol turnover and PKC activity in sponge cells increase. However, specific AR/AF binding occurs for only 5 hours. After that the active form of the AR is phosphorylated by protein kinase C to become inactive (Gramzow et al., 1988). Sponge cells need a second system to maintain cell aggregation after AR inactivation. It is interesting to note one possible difference between sponge cell aggregation and mussel hemocyte aggregation: since PMA could overcome the inhibitory effect of caffeine to induce hemocyte aggregation in my study, the putative adhesion molecule on hemocytes might require (PKC) phosphorylation to activate it. That is, the role of PKC in these two cell systems may be opposite.

PMA could not only overcome the inhibitory effect of caffeine, but also override the $NaIO_4$ effect. Since PMA could partially overcome inhibition due to $NaIO_4$, but not that due to NEM or EDTA, it would appear that $NaIO_4$ probably affects signal receptor molecules, whereas NEM or EDTA may affect actual adhesion molecules involved in hemocyte aggregation and adhesion.

Table IV-1. The Effect of Verapamil on Hemocyte Aggregation and Adhesion.

Assay	Adhesion				Aggregation		
Treatment	Exp1	Exp2	Exp3	S	Exp1	Exp2	Exp3
CMTBS	100.0	100.0	100.0	100.0 ± 0.0	2°	2°	2°
EGTA (15 mM)	6.7	52.2	7.7	22.2 ± 26.0	0°	0°	0°
verapamil (1 mM)	15.7	75.3	39.1	43.4 ± 30.0	0°	1°	0°
verapamil (0.1 mM)	90.0	98.4	-	94.2 ± 5.9	2°	2°	-

Adhesion: the unit is percentage (%) of cells sticking to plastic. The value in CMTBS is treated as 100 % control.

S: represents the mean ± SD of three values of cell adhesion.

Aggregation: 0°, no aggregation; 1°, weak aggregation; 2°, cohesive aggregation.

Figure IV-1a

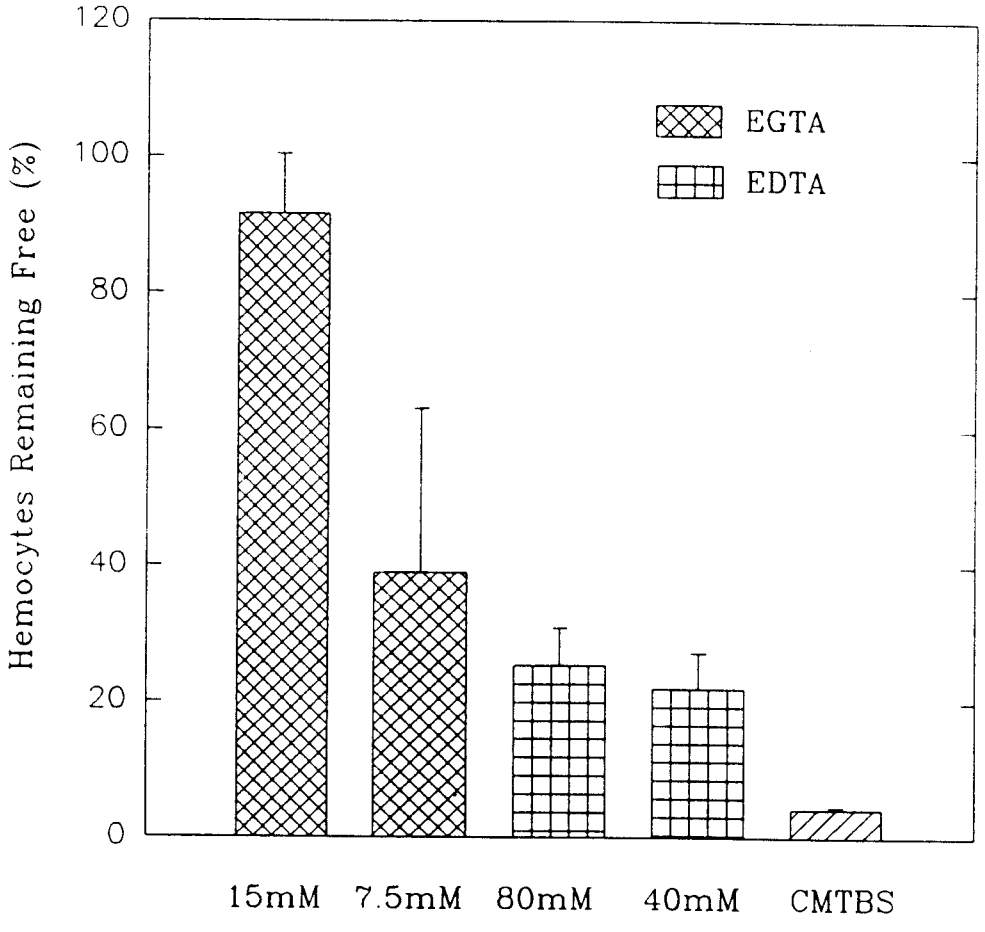


Figure IV-1. Inhibitory effects of EGTA and EDTA on <u>Mytilus californianus</u> hemocyte aggregation and adhesion.

Figure IV-1a. Inhibitory effects of EGTA and EDTA on hemocyte aggregation. Hemocyte aggregation was more strongly inhibited in 15 mM EGTA solution than in 7.5 mM EGTA ($p<0.05$). The 100 % of inhibition index values were obtained from samples fixed in 10 % formalin before they could aggregate.

Figure IV-1b

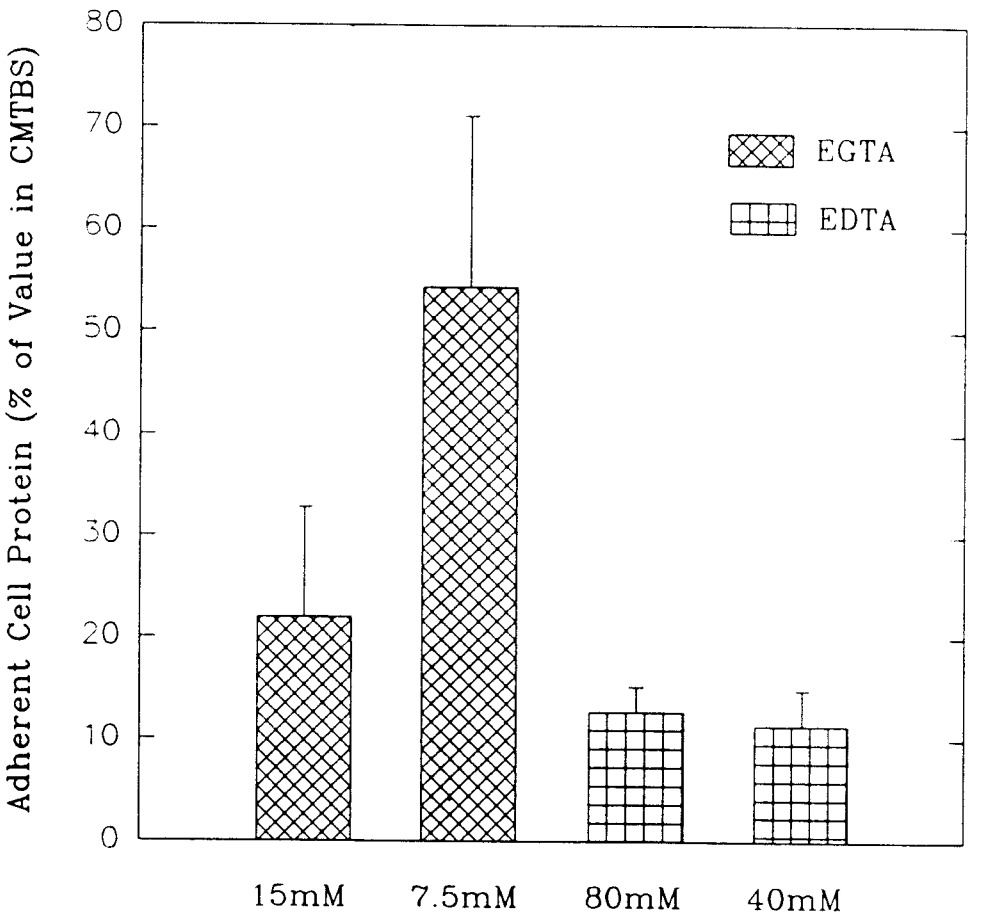


Figure IV-1b. Inhibitory effects of EGTA and EDTA on hemocyte adhesion. In the adhesion assay, 80 mM EDTA or 40 mM EDTA were significantly inhibitory ($p<0.05$). EGTA weakly inhibited hemocyte adhesion at 15 mM, but the effect at 7.5 mM was not significant. The values for cell adhesion in CMTBS were taken as 100 % of control.

Figure IV-2

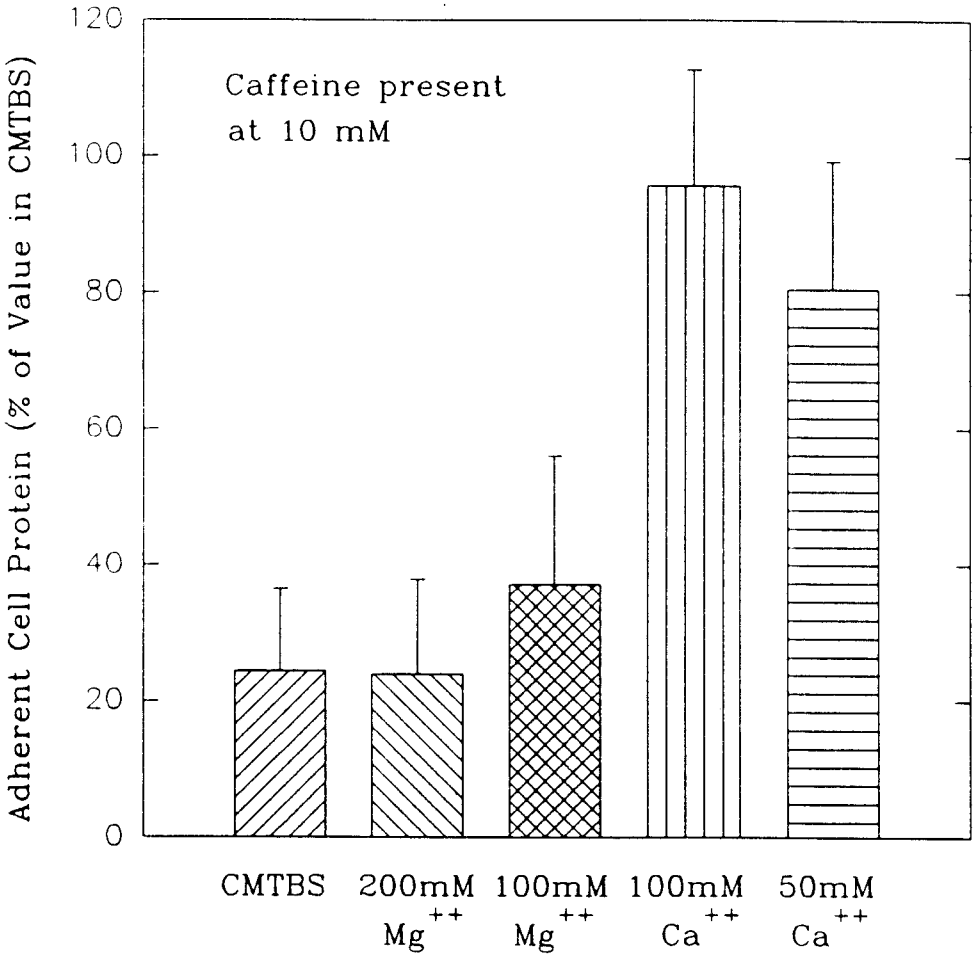


Figure IV-2. The effect of calcium and magnesium on <u>Mytilus californianus</u> hemocyte adhesion in caffeine solution. When calcium concentration in hemolymph was increased to 50 mM or 100 mM $CaCl_2$, the inhibitory effect of 10 mM caffeine on hemocyte adhesion was lost. The values in 50 mM and 100 mM Ca^{++} were not statistically different ($p<0.05$). Magnesium did not overcome caffeine inhibition.

Figure IV-3

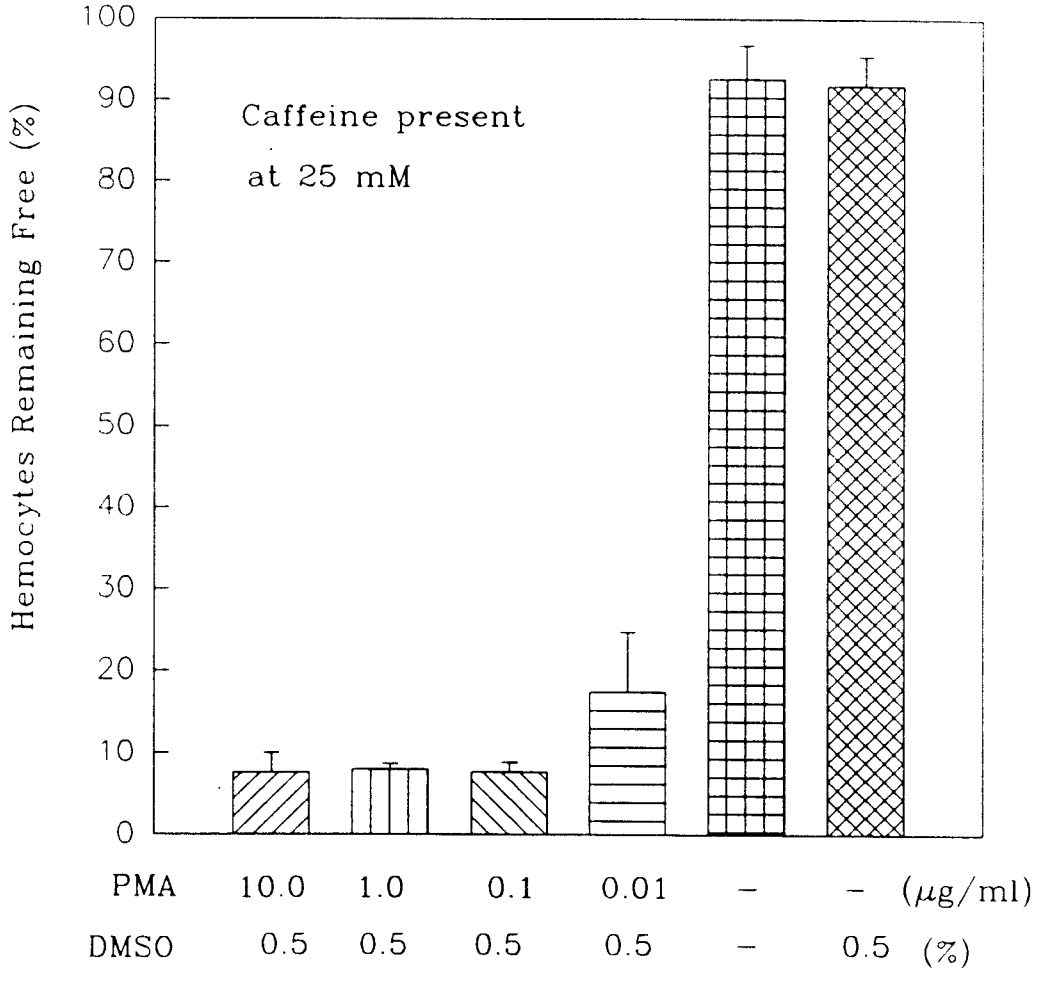


Figure IV-3. The ability of PMA to overcome the inhibition of <u>Mytilus californianus</u> hemocyte aggregation due to 25 mM caffeine. In 25 mM caffeine more than 90 % of hemocytes remained free. The percentage of free hemocytes significantly decreased in caffeine+PMA solutions ($p<0.05$). The solvent DMSO at 0.5 % was without effect.

Figure IV-4

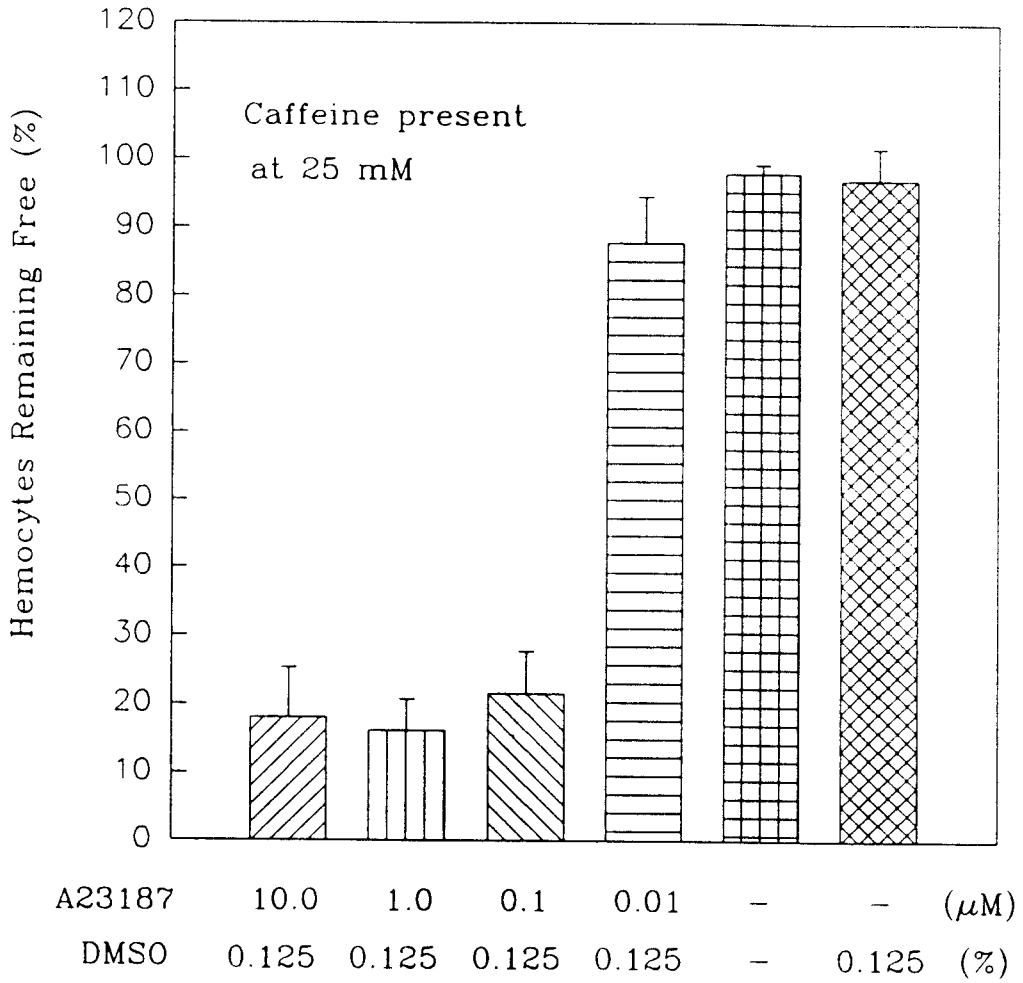


Figure IV-4. The ability of A23187 to overcome the inhibition of Mytilus californianus hemocyte aggregation due to 25 mM caffeine. At concentrations of 0.1 μM or more, A23187 overcame the inhibitory effect of 25 mM caffeine on hemocyte aggregation ($p<0.05$). Only about 20 % of cells remained free when A23187 was present at concentrations > 0.1 μM. At 0.125 % DMSO was without effect.

Figure IV-5

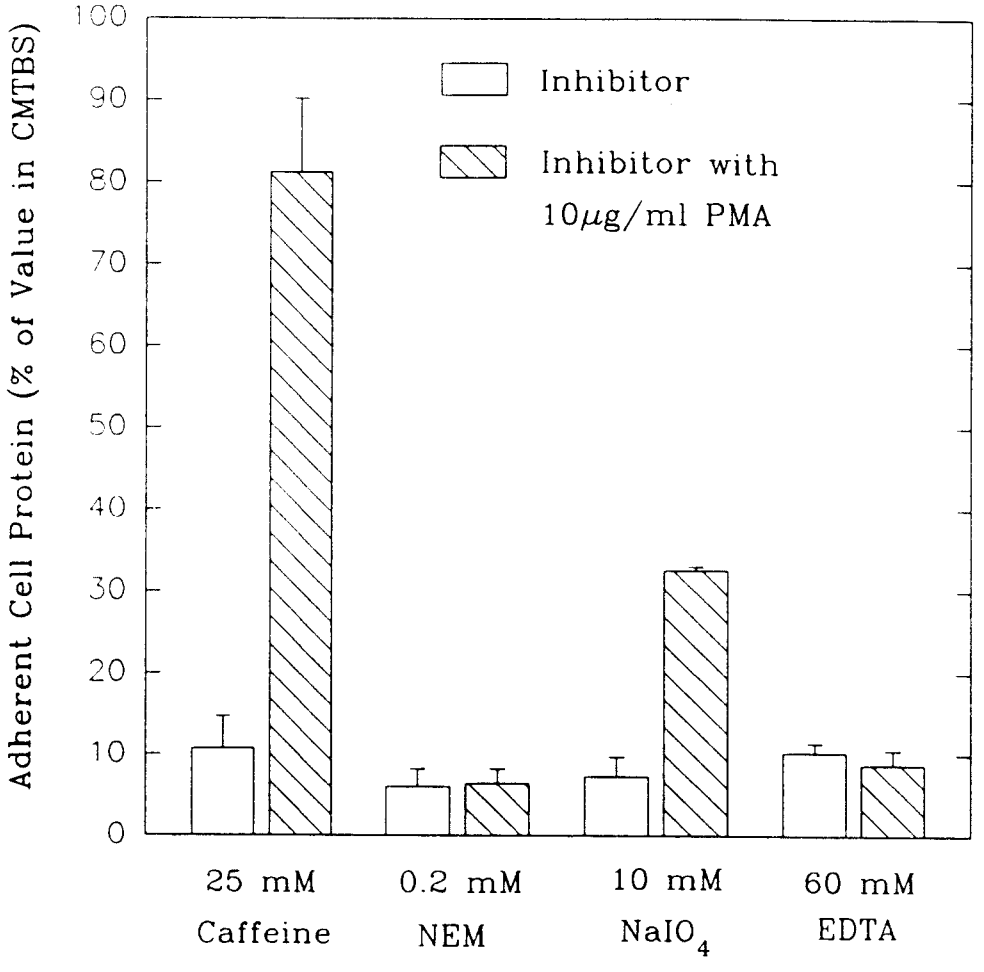


Figure IV-5. The effect of PMA on <u>Mytilus californianus</u> hemocyte adhesion in caffeine, EDTA, NEM, or $NaIO_4$ solution. PMA strongly overrode the inhibition due to caffeine ($p<0.05$), weakly overrode that due to $NaIO_4$ ($p<0.05$), and did not affect inhibition due to EDTA or NEM.

Figure IV-6a

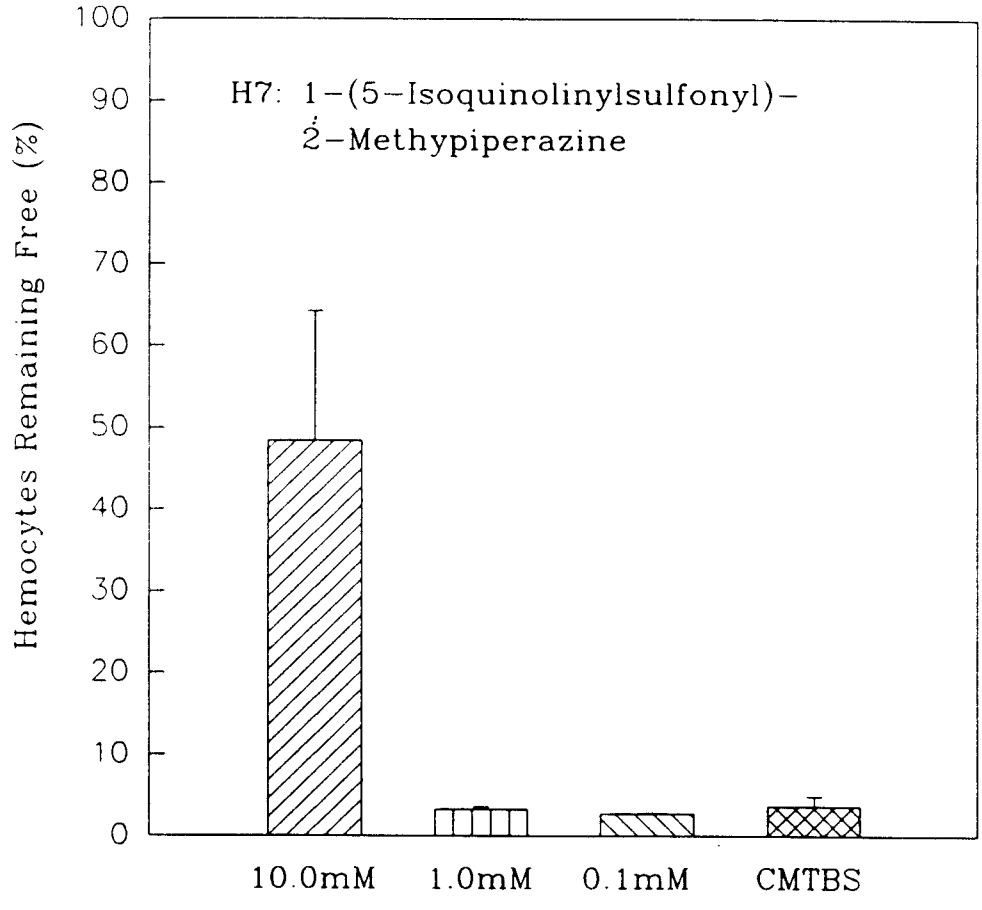


Figure IV-6. The effect of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) on <u>Mytilus californianus</u> hemocyte aggregation and adhesion.

Figure IV-6a. Inhibitory effect of H7 on hemocyte aggregation. No inhibition of hemocyte aggregation was seen in H7 concentrations < 1 mM. However, H7 at 10 mM significantly decreased hemocyte aggregation by 50 % ($p<0.05$).

Figure IV-6b

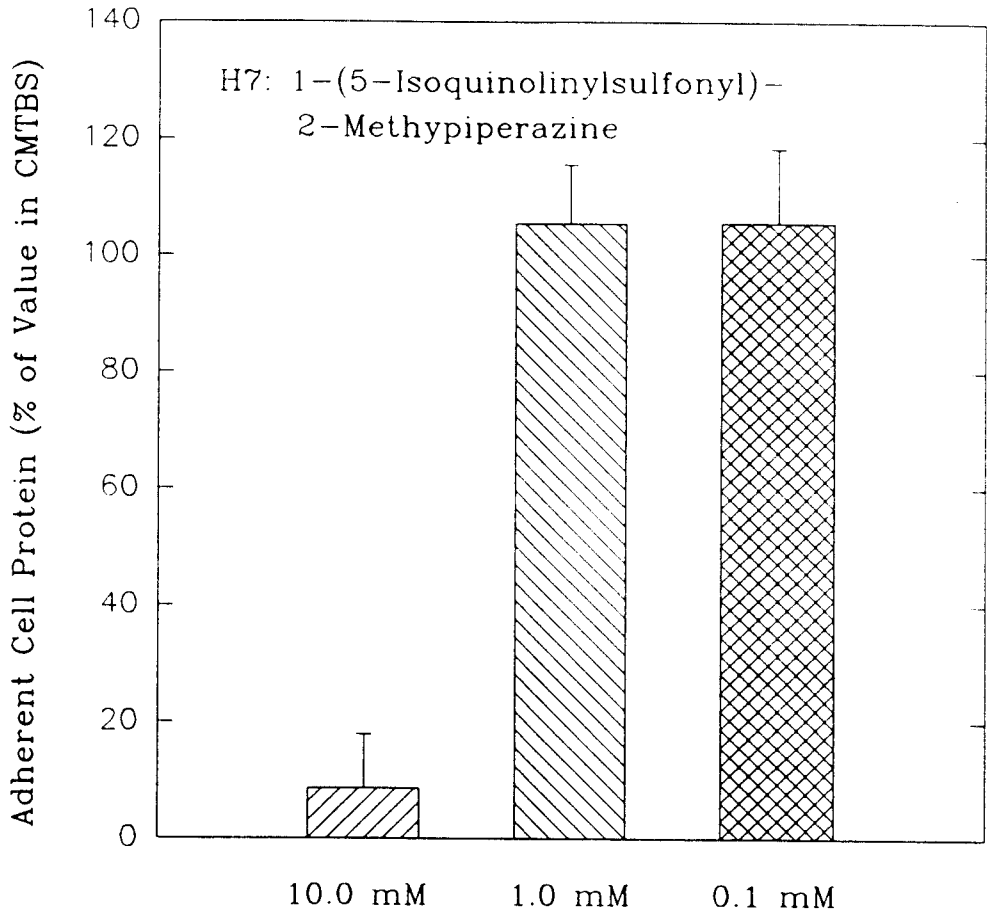


Figure IV-6b. Inhibitory effect of H7 on hemocyte adhesion. At 10 mM, H7 strongly inhibited hemocyte adhesion ($p<0.05$). Less than 10 % cells could adhere. However, no inhibition of hemocyte adhesion was seen at concentrations of H7 < 1 mM.

CHAPTER V

THE APPROPRIATE ROLES OF CARBOHYDRATES IN AGGREGATION AND ADHESION OF HEMOCYTES FROM THE CALIFORNIA MUSSEL (*Mytilus californianus*).

ABSTRACT

A number of enzymes have been found to have differing effects on hemocyte aggregation and adhesion *in vitro*. N-acetyl-glucosaminidase (5 unit/ml) inhibited hemocyte aggregation and adhesion, implicating N-acetyl-glucosamine as a component of ligands involved in cell-cell and cell-substratum interactions. Trypsin partially inhibited hemocyte adhesion at 2.5 mg/ml, but had no effect on hemocyte aggregation. In contrast, hyaluronidase or neuraminidase at concentrations of 5 mg/ml or 2.5 unit/ml respectively did not inhibit either aggregation or adhesion, but enhanced them. Neuraminidase at 2.5 unit/ml elicited weak hemocyte aggregation even in the presence of an inhibiting concentration (25 mM) of caffeine. Hemocytes treated with 25 mM caffeine with or without hyaluronidase or N-acetyl-glucosaminidase could not form aggregates. Inhibition of hemocyte aggregation and adhesion by the calcium channel blocker verapamil was markedly overcome by 5 mg/ml hyaluronidase. Trypsin enhanced the verapamil effect.

Hemocytes were unable to aggregate and adhere in low pH solution. This inhibition could be overcome by PMA. Therefore, the action of low pH on cell behavior appears to be due to its effect on an early stage of activation.

INTRODUCTION

The involvement of extracellular domains of integral membrane proteins in cell-cell recognition and adhesion has become clear during the past 10 years (Hynes, 1987; Springer, 1990). Due to the variety of constituent amino acids, proteins were formerly considered to be the molecules most likely to be responsible for such specific interactions. Carbohydrates, the other main components expressed extracellularly, were formerly neglected as possible ligands for cell-cell interactions. Most cell membranes contain glycoproteins and glycolipids, the carbohydrate moieties of which are located on their extracellular terminals to form an outer carbohydrate layer, the glycocalyx. Carbohydrate components of glycoproteins or glycolipids can be complex due to a number of features including their branched structure and residue sequence. Also, sulfates, phosphates and acetyl groups add diversity to the carbohydrate moieties further increasing their structural complexity (reviewed by Brandly & Schnaar, 1986). Although the basis of molecular complexity in carbohydrates differs from that of protein, unique carbohydrate structures do mark specific cell membranes and are consequently involved in cell-cell interaction. On the other hand, the glycocalyx may prevent protein-protein interactions between cells (Rutishauser et al., 1988). In addition, sialic acid, a major carbohydrate component on cell membranes, generates a negative charge on cell surfaces causing repulsive forces between cells (Greig & Jones, 1977; Bell, 1983).

The other evidence of carbohydrate involvement in cell interactions is obtained from studies focussed on lectins (Yeaton, 1981; Brandley & Schnaar, 1986; Renwrantz, 1986). Lectins are a group of proteins that bind specifically with carbohydrate components of glycoproteins, glycolipids or pure carbohydrates (Lis & Sharon, 1986). Lectins have been known

to be involved in cell-cell interactions including slime mold differentiation (Rosen et al., 1976; Barondes et al., 1978), sponge cell reaggregation (Bretting et al., 1976; Varner et al., 1988), human blood clotting (Gartner et al., 1981; Jaffe et al., 1982), lymphocyte recirculation (Ashwell & Harford, 1982; Streeter et al., 1988), species-specific binding of sperm to egg (Vacquier & Moy, 1977; Rosati & de Santis, 1980), pathogen-host adhesion (Jungery et al., 1983; Villata & Kierszenbaum, 1983; Ward et al., 1987), virus infection (Varghese et al., 1983; Mitsuya et al., 1988), and bacterial adhesion (Eshdat et al., 1978). In molluscs and arthropods, an opsonic role for lectins in hemocyte phagocytosis has been reported (Renwrantz, 1986; Bayne, 1990).

Although no inhibitory activity was seen from more than 50 sugars which were used in efforts to inhibit hemocyte aggregation and adhesion (Chapter 2), the possible involvement of carbohydrates in hemocyte aggregation and adhesion was not excluded. Not only are hemocyte surface molecules recognized by specific lectins (Richards et al., 1989, Pipe, 1990), but also native lectins have been found on hemocyte surfaces (Vasta et al., 1982; Richards & Renwrantz, 1990).

Solutions of monosodium glucose-6-phosphate (G-6-P, Na^+) did inhibite hemocyte aggregation and adhesion at 200 mM (Chapter 2). However, the pH of the 200 mM G-6-P treated hemolymph was acidic. Therefore, the inhibitory effect of G-6-P may have been due to its acidic nature. In order to further verify the inhibitory activity of G-6-P on hemocyte activation, several analogues of G-6-P have been tested.

MATERIALS AND METHODS

Reagents

N-acetyl glucosaminidase (EC 3.2.1.30), neuraminidase (sialidase; EC 3.2.1.8; type V), hyaluronidase (EC 3.2.1.35;

type IV-S), trypsin (EC 3.4.21.4; type IX), and other reagents were purchased from Sigma Co.. Solutions of enzymes, caffeine, verapamil, monosodium glucose-6-phosphate, disodium glucose-1-phosphate (G-1-P, $2Na^+$), and disodium mannose-6-phosphate (M-6-P, $2Na^+$) were prepared fresh on the day of use.

Enzymatic treatment of hemolymph

The protocol of enzymatic treatment of hemolymph is same as that of other chemical treatments. Briefly, the enzyme was freshly dissolved in CMTBS at the appropriate concentration. The volume ratio was 1:1 of enzyme solution to hemolymph, or 1:1:2 of enzyme solution to inhibitor (caffeine or verapamil) and to hemolymph.

Hemocyte adhesion and aggregation assay

The hemocyte adhesion and aggregation assay were described in Chapter 2. The level of hemocyte aggregation is reported as no aggregation (0°), weak aggregation (1°), or cohesive aggregation (2°).

Cell viability

Following the assays for adhesion and spreading, and for aggregation, hemocyte viability was determined by cell exclusion of propidium iodide. The propidium iodide staining protocol was as follows: 1 μl propidium iodide (500 μg/ml in CMTBS) was added to each sample (50 μl). In cells which are dead, the dye enters and binds to nuclear DNA with high affinity. Stained nuclei generate bright red fluorescence under epi-illumination using a Zeiss microscope with a set of filters appropriate for rhodamine isothiocyanate.

Data analysis

Each experiment was repeated at least three times, using fresh hemolymph samples from different animals. Data are presented as mean ± S.D.. Statistical analysis of data was

performed using paired or unpaired Student's t-test as appropriate. Differences were considered significant when $p<0.05$.

RESULTS

Effects of enzymes on hemocyte aggregation

N-acetyl-glucosaminidase at 5 units/ml interfered markedly with hemocyte aggregation (Table V-1). Since the enzyme was originally dissolved in 3.2 M ammonium sulfate buffer solution, the possible effect of ammonium sulfate was tested. No obvious effect on aggregation was seen. Three other enzymes including trypsin, hyaluronidase, and neuraminidase failed to influence hemocyte aggregation. Interestingly, when 2.5 units/ml neuraminidase were mixed with 25 mM caffeine and used to treat hemocytes, weak aggregates were formed. Hemocytes treated with 25 mM caffeine with or without 5 mg/ml hyaluronidase or 5 unit/ml N-acetyl-glucosaminidase, however, did not aggregate. Similar results were seen when verapamil was added to the enzyme solutions. At 5 mg/ml, hyaluronidase significantly overcame the inhibitory effect of verapamil to induce cohesive aggregation. Such aggregation was not seen in other enzyme+verapamil or verapamil treatments.

Effects of enzymes on hemocyte adhesion

In hemocyte adhesion assays, N-acetyl-glucosaminidase at 5 unit/ml with 320 mM ammonium sulfate had significant inhibitory activity. Although adhesion was retarded in the 320 mM ammonium sulfate without enzyme, treated hemocytes were spread out after 10 minutes (data not shown). Trypsin reduced hemocyte adhesion to 60 % of control values. Hemocyte adhesion was not affected by 5 mg/ml hyaluronidase or 2.5 unit/ml neuraminidase (Fig.V-1). However, 5 mM hyaluronidase almost completely overcame inhibition due to 1 mM verapamil treatment

(Fig.V-2). Inhibition due to 25 mM caffeine was not overcome by 5 mg/ml hyaluronidase, 2.5 unit/ml neuraminidase or 5 unit/ml N-acetyl glucosaminidase (data not shown).

Effect of low pH on hemocyte adhesion

The pH value of 50 mM monosodium glucose-6-phosphate in hemolymph is a little above 5.0. In this acidic condition, the extent of inhibition of hemocyte adhesion was variable. Cell viability, checked by propidium iodine exclusion, was still more than 90 % after 30 minutes. However, glucose-6-phosphate at 25 mM which has a pH near 5.5 was almost unable to inhibit hemocyte adhesion. The inhibition observed in 50 mM glucose-6-phosphate disappeared when the acidic solution was neutralized to pH 7.4 before being mixed with hemolymph. Similarly, 50 mM disodium glucose-6-phosphate, which has a pH value around 7.0 in hemolymph, failed to inhibit hemocyte adhesion. Also, no inhibitory effect was seen in 50 mM disodium glucose-1-phosphate and disodium mannose-6-phosphate (Fig.V-3).

Hemocyte activities that were inhibited in acidic G-6-P (Na^+) were activated when phorbol ester (PMA) was also present (Fig.V-4). The inhibitory effect of low pH was reduced when hyaluronidase was present at either 5 mg/ml or 1 mg/ml (Fig.V-5). However, the effects of neuraminidase varied between experiments.

DISCUSSION

Since all known cell adhesion molecules, whether involved in cell-cell adhesion or cell-substratum adhesion, are proteins, it was to be expected that trypsin would affect hemocyte adhesion. Therefore it was surprising that trypsin only partially reduced hemocyte adhesion, and had no effect on hemocyte aggregation. A possible explanation for this is

inactivation of added trypsin by endogenous protease inhibitors. Bender et al. (1991) have reported that endogenous protease inhibitors exist in the hemolymph of the fresh water snail, Biomphalaria glabrata. Weak protease inhibiting activity has been found in Mytilus californianus hemolymph (data not shown). A reasonable alternative explanation for the inability of trypsin to affect mussel hemocyte aggregation is that the hemocyte aggregation is so rapid that the trypsin would not have had sufficient time to hydrolyze the adhesion molecules.

N-acetyl glucosaminidase markedly reduced hemocyte aggregation and adhesion. Mytilus edulis hemocytes contain N-acetyl glucosamine on their surfaces (Pipe, 1990). Thus, N-acetyl glucosamine may play an important role in hemocyte adhesion and aggregation. The failure to observe any inhibitory effect of 200 mM N-acetyl glucosamine added to hemolymph (see chapter 2) may be accounted for if those features of the adhesion molecules that are recognized are intramolecular linkages or large domains, rather than component monosaccharides.

Hyaluronidase and neuraminidase on their own failed to affect hemocyte aggregation and adhesion. However, when they were individually mixed with verapamil or caffeine, their enhancing effects were apparent. In previous studies (Chapter 4), the inhibitory effect of caffeine was overcome by PMA. Since it is known that this protein kinase C activator can cause sialic acid loss from cell surfaces (Nabi et al., 1989), I suspected that the removal of sialic acid might enhance hemocyte aggregation and adhesion. When treated with caffeine+neuraminidase, hemocytes retained weak aggregation competence, but there was no significant change in hemocyte adhesion. Since, unlike PMA, neuraminidase did not enhance free hemocytes to form cohesive aggregates in caffeine solution, it appears that the removal of sialic acid is not sufficient to cause cohesive aggregation. Desialization must

have to be coupled with other cellular processess such as cytoskeleton assembly (Chapter 2). On the other hand, sialic acid removal is not essential for hemocyte adhesion. This identifies another characteristic that distinguishes hemocyte aggregation and adhesion.

Hyaluronate involvement in cell aggregation has been thoroughly studied by Underhill and his colleagues (Underhill & Dorfman, 1978; Underhill, 1982; Green et al., 1988), and a specific hyaluronate receptor has been isolated and characterized (Underhill et al., 1985). Cell surface hyaluronates are important for both homologous (Underhill & Dorfman, 1978) and heterologous cell interactions (Green et al., 1988). However, in Mytilus, hyaluronic acid seems to play an opposite role. Its removal seems to permit mussel hemocyte adhesion and aggregation to occur. When the hemocytes were treated with verapamil, a Ca^{++} channel blocker, hemocyte aggregation and adhesion were interrupted. But 5 mg/ml hyaluronidase completely reversed the inhibition due to 1 mM verapamil. The mechanism is not understood. However, this could explain why hyaluronidase was not inhibitory, but rather slightly enhanced hemocyte adhesion (Fig. V-1).

The aggregation of membrane vesicles from chicken embryonic brain cells that express neural cell adhesion molecules (N-CAM) was increased by an endoneuraminidase from phage (Rutishauser et al., 1985). Neural cell-cell adhesion was increased when the expressed N-CAM was low in polysialic acid (PSA) content (Rutishauser et al., 1988). By analogy, cell surface carbohydrates that change during cell activation may influence with cell-cell adhesion. The N-CAM interaction model may be applicable to the Mytilus hemocyte system. Accordingly we might suggest that the glycocalyx in resting hemocytes differs from that in activated cells, where this carbohydrate-rich structure may be modified by endogeneous carbohydrate-specific enzymes. But the reality of this, and the mechanisms of action of these putative enzymes and their

activation remain to be discovered.

Based on the failure of neutralized G-6-P (pH 7.4) and disodium salt G-1-P (pH 7.0) to inhibit hemocyte adhesion and aggregation, I postulated that the inhibitory effect of monosodium G-6-P (ph 5.0) was due to its acidic character, not to its structure. Similarly, M-6-P (pH 7.0) did not show any inhibitory effect on hemocyte adhesion and aggregation. Though lectins specific for mannose-6-phosphate have been isolated from mammalian cells (Simpson et al., 1978), and though these lectins regulate specific cell activity (Sahagian, 1984), M-6-P is evidently not associated with Mytilus hemocyte activation. Interestingly, 25 mM G-6-P (pH 5.5) did not inhibit hemocyte adhesion like 50 mM G-6-P (pH 5.0). This implies that the pH value is very criticalin the inhibitory effect.

Phorbol ester has been found to overcome the inhibitory effect of caffeine (Chapter 4). In this study, cells retained adhesion competence in acidic G-6-P (pH 5.0) when phorbol ester was present. The drug has been widely used as a cell activator via protein kinase C activation. However, it was also reported that phorbol ester can influence cytoplasmic calcium concentrations (Grinstein & Goetz, 1985), and alter cytoplasmic pH (Moolenaar et al., 1984; Grinstein et al., 1985). Therefore, the regulation of cytoplasmic pH in Mytilus hemocytes may be the other control mechanism of cell activation. That is, the cytoplasmic pH of resting hemocytes may be maintained below 7. When the cells are activated, the cytoplasmic pH rapidly shifts from acidic to basic, thus regulating pH-sensitive cellular activities.

Table V-1. The effects of various enzyme treatments on Mytilus californianus hemocyte aggregation.

Treatment	Hemocyte aggregation level*		
	Exp 1	Exp 2	Exp 3
trypsin**	2°	2°	2°
hyaluronidase	2°	2°	2°
neuraminidase	2°	2°	2°
N-acetyl glucosaminidase	0°	1°	0°
caffeine***	0°	0°	0°
hyaluronidase/caffeine	0°	0°	0°
neuraminidase/caffeine	1°	1°	1°
N-acetyl glucosaminidase/ caffeine	0°	0°	0°
verapamil***	0°	0°	1°
trypsin/verapamil	0°	0°	1°
hyaluronidase/verapamil	2°	2°	2°

* Hemocyte aggregation levels: 0°, no aggregation; 1°, weak aggregation; 2°, cohesive aggregation.

** The concentrations of enzymes in these experiments: trypsin, 2.5 mg/ml; hyaluronidase, 5 mg/ml; neuraminidase, 2.5 unit/ml; N-acetyl glucosaminidase, 5 unit/ml.

*** In these experiments, the caffeine concentration was 25 mM; and the verapamil concentration was 1 mM.

Figure V-1

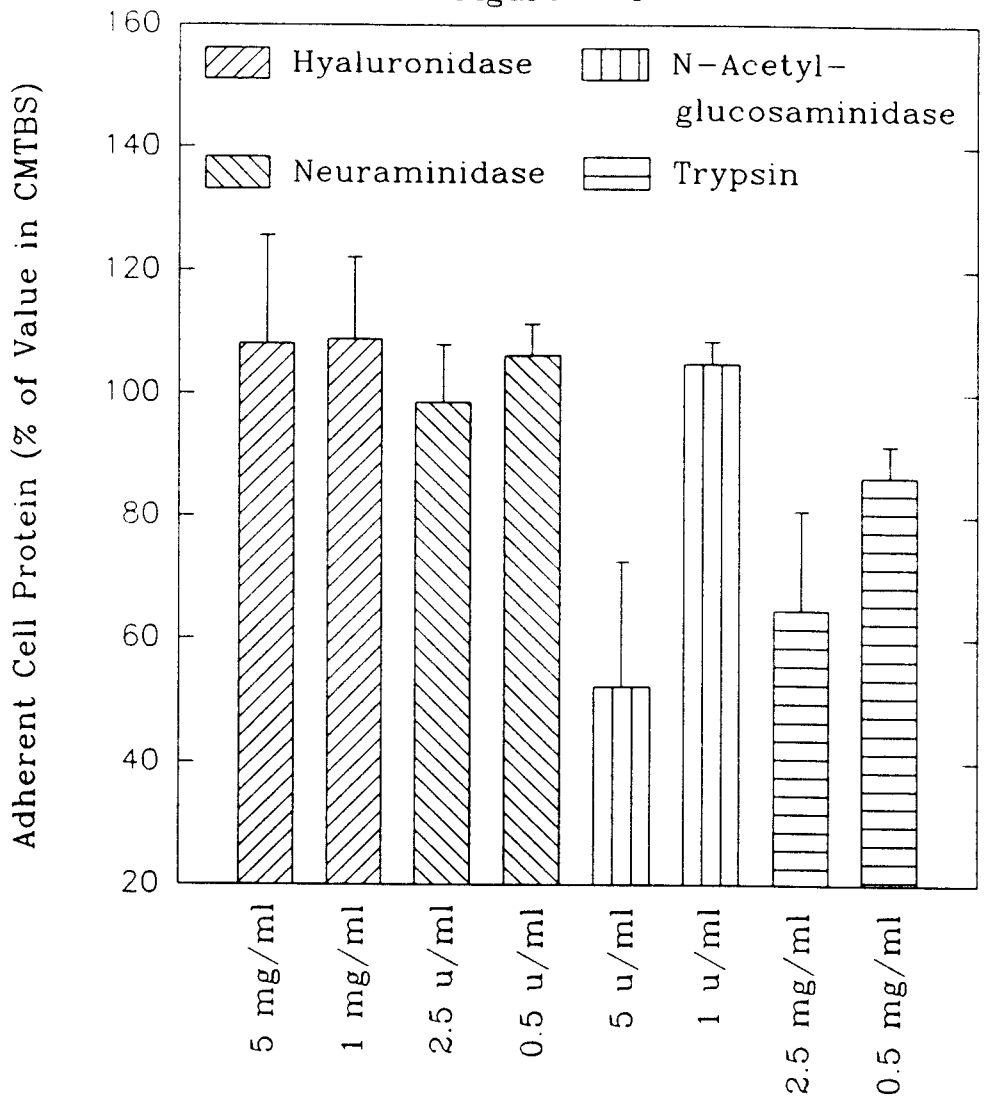


Figure V-1. The effects of enzymes on <u>Mytilus californianus</u> hemocyte adhesion. N-acetyl glucosaminidase (5 unit/ml) with 320 mM $(NH_4)_2SO_4$ significantly inhibited hemocyte adhesion ($p<0.05$). Trypsin at 2.5 mg/ml reduced hemocyte adhesion to 60 % of the control value obtained in CMTBS ($p<0.05$). Hyaluronidase and neuraminidase did not inhibit adhesion.

Figure V-2

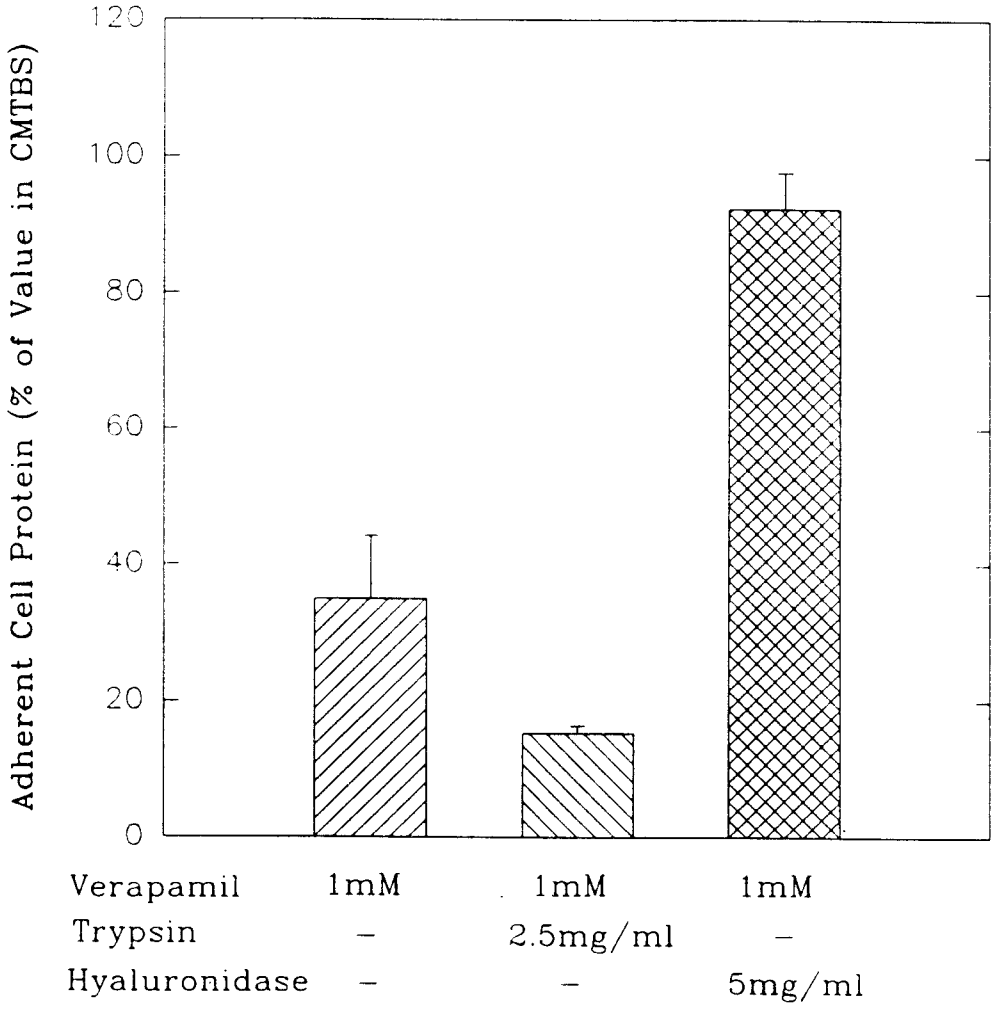


Figure V-2. The effect of verapamil+enzyme mixture on <u>Mytilus californianus</u> hemocyte adhesion. The hemocytes failed to adhere and spread in 1 mM verapamil treatment, but the cells adhered and spread well in verapamil when 5 mg/ml hyaluronidase was also present ($p<0.05$). Trypsin at 2.5 mg/ml did not have this effect.

Figure V-3

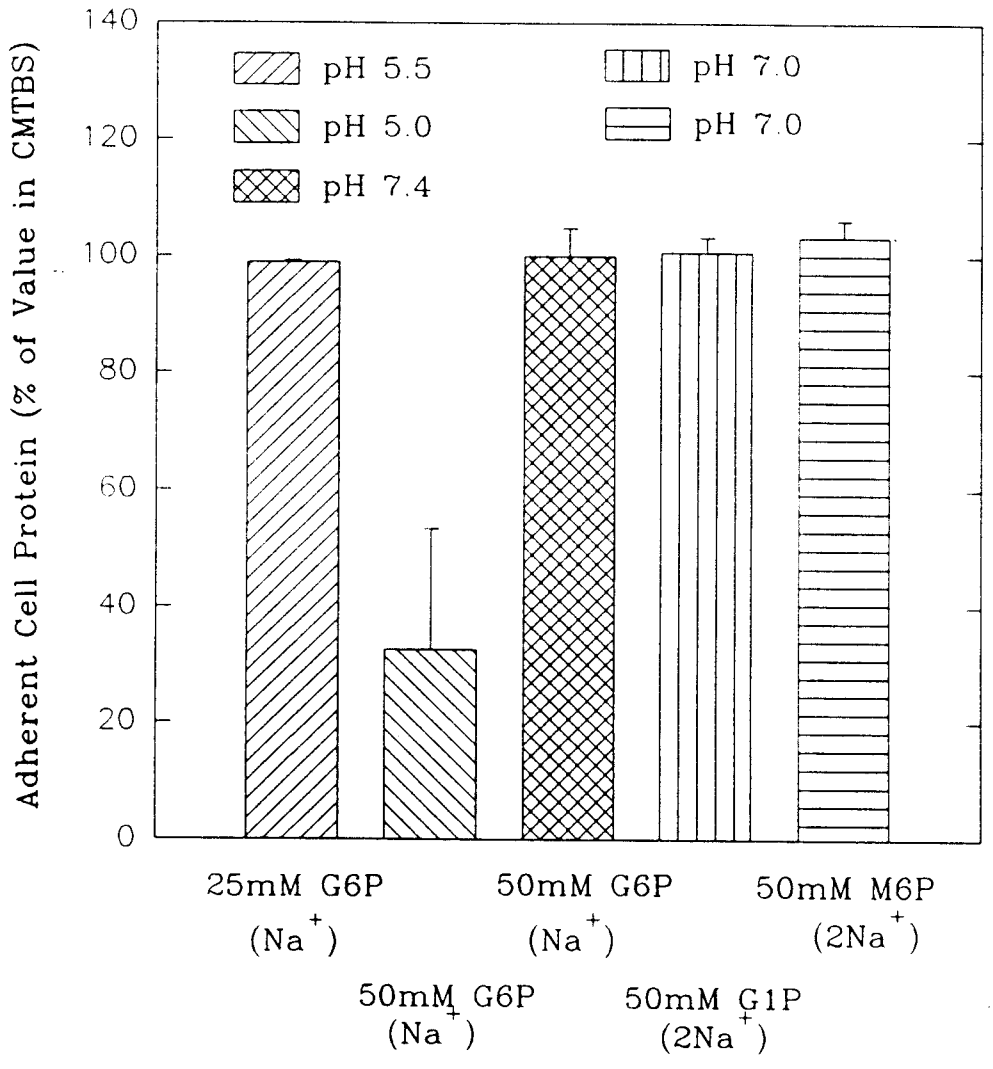


Figure V-3. The effects of glucose-6-phosphate and its analogues on <u>Mytilus californianus</u> hemocyte adhesion. The hemocytes were unable to adhere in 50 mM G-6-P (pH 5) ($p<0.05$), but they could adhere and spread well in 25 mM G-6-P (pH 5.5). However, in the neutralized (pH 7.4) G-6-P solution and in disodium G-1-P and M-6-P solution, which at 50 mM concentration in hemolymph have pH values near 7.0, hemocyte adhesion was not affected.

Figure V-4

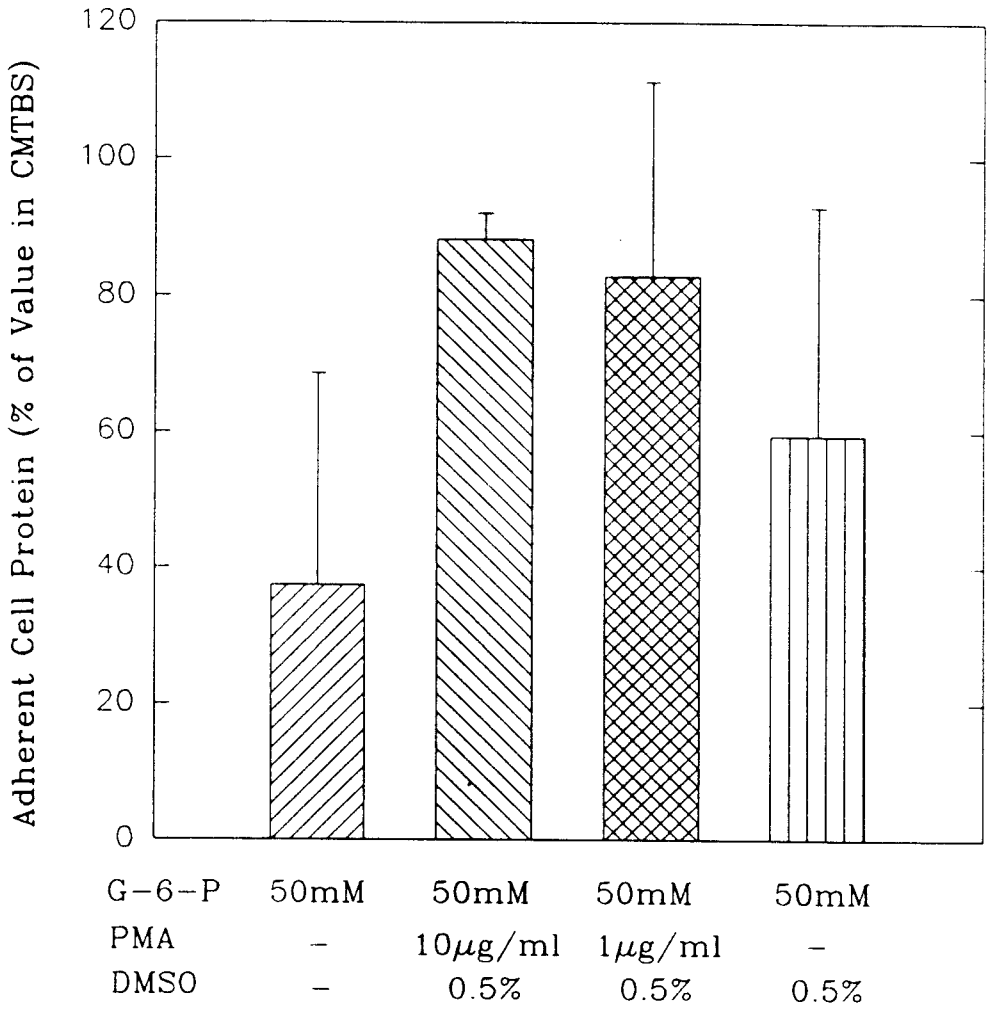


Figure V-4. The ability of PMA to overcome inhibition of Mytilus californianus hemocyte adhesion in glucose-6-phosphate solution. PMA (10 μg/ml) significantly enhanced hemocyte adhesion in 50 mM G-6-P solution (pH 5.0) ($p<0.05$). PMA (1μg/ml) and DMSO, the solvent of PMA, at 0.5 % did not promoted hemocyte adhesion in the same G-6-P solution.

Figure V-5

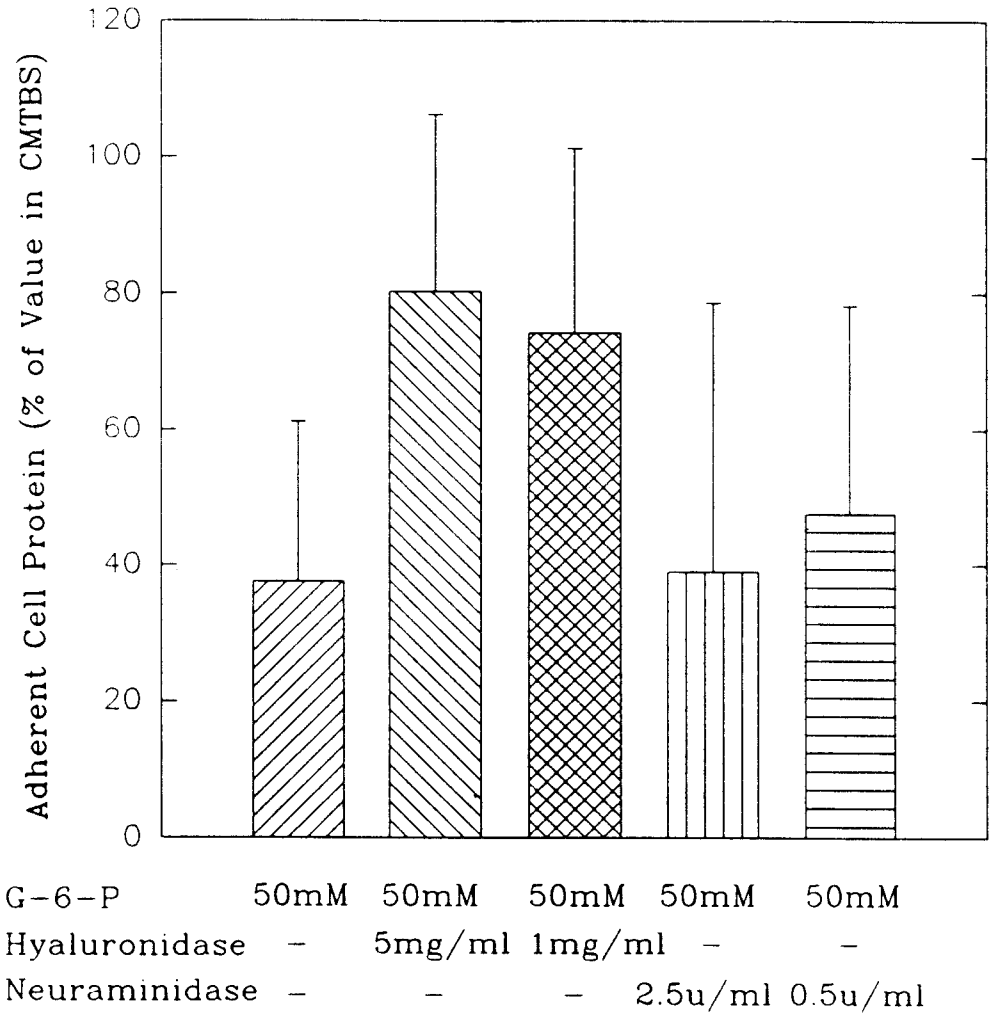


Figure V-5. <u>Mytilus californianus</u> hemocyte adhesion in glucose-6-P+enzyme solution. More hemocytes adhered in G-6-P+hyaluronidase (5 mg/ml) than in G-6-P alone (paired t-test, $p<0.05$). The effect of neuraminidase in similar experiments varied.

CHAPTER VI

CONCLUSION

When hemolymph (blood) is removed from a mussel, the hemocytes rapidly aggregate and will adhere to foreign surfaces. These behaviors are essential components of natural defenses, hemostasis and wound healing. We use these behaviors as indicators of hemocyte activation. Hemocyte activation in molluscs has been studied to only a very limited extent. In sponges, cell aggregation has been extensively studied as a model of cell recognition and differentiation. T cell activation is the other well known model for study of cell recognition and cell communication. Data from all three model systems imply that mechanisms of cell activation are highly conserved throughout evolutionary history.

To understand the mechanisms operating during cell aggregation, several cell models including slime molds, sponges, myoblasts, neural cells, platelets, and immune cells have been studied. Although aggregation in these diverse systems occurs in response to different stimulators and involves different adhesion molecules, the intracellular signal transduction systems and some regulatory systems are similar. In these cell models, aggregation processes have been reported as two- or three-step events. For example, in sponges, the first step of aggregation is cell-cell recognition which is divalent cation- and soluble aggregation factor-independent. However, the second step, secondary aggregation, is divalent cation- and soluble aggregation factor-dependent. Mussel hemocyte aggregation is also a two-step reaction, and is controlled by temperature, divalent cations and metabolic energy.

In other model systems cell adhesion molecules (or named aggregation receptors) have been isolated and characterized;

some are Ca^{++}-dependent and others are Ca^{++}-independent. There can be homophilic (identical molecules bind to one another) or heterophilic (binding between different molecules) binding. Although the adhesion molecules on mussel hemocyte surfaces have not been identified, indirect evidence reveals that cell surface proteins and carbohydrates are involved. These putative adhesion molecules are sensitive to enzymes such as trypsin, neuraminidase, hyaluronidase, and N-acetyl-glucosaminidase. This accords with data from other systems indicating that cell surface carbohydrates are important in cell aggregation. However, in different systems carbohydrates have contrasting roles: some are required for cell-cell recognition and binding whereas others prohibit cell-cell contact. For example, sialic acid residues of certain cell surface glycoproteins possess a negative charge that can generate repulsion forces to avoid cell-cell interaction. Some cell surface enzymes have been considered to be cell activation promoters that work by removing a shield portion from adhesion molecules to expose active binding domains. In M. californianus hemocytes, neuraminidase and hyaluronidase promoted cell aggregation even when inhibitor was present, but N-acetyl-glucosaminidase was inhibitory. Therefore, N-acetyl-glucosamine may be essential for mussel hemocyte aggregation, whereas sialic acid and hyaluronic acid may be important in maintaining a resting state in these cells.

Cell adhesion to foreign surfaces is a non-self recognition process. An aim of this thesis was to reveal hemocyte mechanisms of such recognition. A variety of potential recognition systems were examined. One of these was the integrin-RGD recognition system. A variety of cell types recognize the tri-amino acid sequence RGD in macromolecules such as fibronectin and laminin. RGD-containing proteins and proteoglycans such as collagen are common components of the extracellular matrix. Such matrices not only anchor cells but also induce cell differentiation. Spreading of mussel

hemocytes was retarded by RGD-containing peptides, but these peptides did not inhibit hemocyte adhesion. On the other hand, mussel hemocyte adhesion, as seen in the adhesion of other cells, was affected by surface charge. In most cases, hemocytes could adhere and spread faster on polystyrene than glass surfaces. Therefore, mussel hemocyte adhesion may be effected by more than one mechanism.

Since adenosine receptors are coupled with adenylate cyclase, adenosine is regarded as an important endogenous inducer capable of initiating cell activation via cAMP generation. Although there is no direct evidence of adenosine receptors on mussel hemocytes, the ability of adenosine and its analogues to compete with caffeine for regulation of hemocyte activation strongly supports our view that adenosine or adenosine-like receptors are present on mussel hemocyte surfaces.

The regulation of cell activation by either the cAMP or the phospholipid/Ca^{++} pathway has been demonstrated in a variety of cell types. Both pathways appear to be operational in mussel hemocytes in controlling aggregation and adhesion. As with many other studies, ours have failed to provide answers to two contemporary questions in cell biology. First, how do cells use one common transduction pathway to precisely transduce signals from distinct receptor types to elicit distinct responses? Secondary, what is the relationship between the cAMP-dependent protein kinase PKA, and the Ca^{++}-dependent protein kinase PKC? As reported in Chapter 3, the inhibitory effect of caffeine appears to be mediated via adenosine receptors which change intracellular cAMP concentration thus affecting protein kinase A activity. However, the inhibitory effect of caffeine was found to be overcome by PMA, generally considered to be a protein kinase C activator. It is not yet clear how the relative activities of protein kinase C and protein kinase A are regulated. And there are no recent data to help us understand the interaction

between adenosine receptors and the phospholipid transduction system. Thus, although both cAMP and the phospholipid transduction system appear to be involved in mussel hemocyte aggregation and adhesion, their relationship remains to be understood. Since both protein kinases appear to be active during cell activation, their respective target proteins may affect each other. Indeed, there is evidence that protein phosphorylation occurred during mussel hemocyte activation (data not shown). However, further research is needed to reveal the nature of protein kinase target proteins.

Since the adhesion molecules on mussel hemocyte surfaces have not yet been identified, these can not be used to distinguish cell adhesion and aggregation. These two cell behaviors reflect changes in the environment and are always activated simultaneously. However, data on the effects of inhibitors used here implied that cell adhesion and cell aggregation are distinct. For example, 6 mM EDTA could completely inhibit hemocyte adhesion, but not inhibit hemocyte aggregation. When the cells encounter each other before they contact the substratum, aggregation occurs first. The aggregated cells then adhere and spread after the cell mass attaches to a substratum. Therefore, the early pathway of signal transduction of these two cell activities is probably the same.

There remain several unresolved questions concerning hemocyte activation in the mussel. However, a model of hemocyte activation can be erected on the basis of what is known. Such a model is presented in Figure VI-1.

The inhibitory effects of cytochalasin B, described in Chapter 2, suggest that the cytoskeleton is coupled to adhesion molecules associated with morphological changes during cell spreading and cohesive aggregation. EDTA affected cell adhesion at concentrations $\geq$ 0.6 mM, however, weak aggregation occurred in 60 mM EDTA. These results suggest that the cell-substratum adhesion molecules are more EDTA-sensitive

than cell-cell adhesion molecules. Cell aggregation of EDTA-treated hemocytes was stopped at the stage of weak aggregation, with no cohesive aggregation unless Ca^{++}/Mg^{++} were present. This implies that weak aggregation is Ca^{++}/Mg^{++}-independent, and cohesive aggregation is Ca^{++}/Mg^{++}-dependent.

Although neither adenosine receptors nor adenylate cyclase were identified in Mytilus hemocytes, indirect evidence, reported in Chapter 3, implies that the signal transduction pathway of cAMP is involved in hemocyte activation. The adenosine analogues which have high binding affinity to A_1 adenosine receptors have a synergistic effect with caffeine, inhibiting cell aggregation and adhesion. In hemocytes treated with such drugs, cAMP levels were lower than in those treated with CMTBS. Since adenylate cyclase activity is inhibited when A_1 adenosine receptors are bound, and is stimulated when A_2 adenosine receptors are bound, this suggests that the inhibitory effect of caffeine is via adenosine receptors on the hemocyte surface. The cytoplasmic cAMP level may reflect the extent of hemocyte activation.

PKC activity was not measured directly in this study. However, four experimental approaches (Chapter 4) provide indirect evidence that phospholipid/Ca^{++} is the other signal transduction pathway in Mytilus hemocytes: 1) H7, a protein kinase C inhibitor, blocked hemocyte aggregation and adhesion. 2) PMA, a PKC activator, caused hemocyte aggregation and adhesion in caffeine. 3) A23187, a calcium ionophore, also caused hemocyte aggregation and adhesion in caffeine. 4) Verapamil (a calcium channel blocker) and EGTA (calcium-specific chelator) inhibited hemocyte aggregation and adhesion. Although the involvement of PKA and PKC in hemocyte activation is suggested, the interactions between PKs and adhesion molecules, as well as the relationship between PKA and PKC are still unknown.

The involvement of sugars in hemocyte aggregation and adhesion was shown in Chapter 5. N-acetyl glucosaminidase,

hyaluronidase, and neuraminidase produced different effects on adhesion and aggregation: 1) N-acetyl glucosaminidase reduced both hemocyte adhesion and aggregation. 2) The removal of hyaluronic acid enhanced hemocyte aggregation and adhesion. 3) The removal of sialic acid promoted hemocyte aggregation. These results suggest that sialic acid and hyaluronic acid may be located near the terminal end and the N-acetyl glucosamine may be located near the membrane in these cell adhesion molecules.

Figure VI-1. A hypothetical model of Mytilus californianus hemocyte activation. For a discussion of this figure, see pages 126 to 128. Abbreviation: AC, adenylate cyclase; PKA, protein kinase A; HA, hyaluronic acid; CAM, cell adhesion molecule; PKC, protein kinase C; A23187, calcium ionophore; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PMA, phorbol 12-myristate 13-acetate; DAG, 1,2-diacylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-triphosphate; 2-CLAD, 2-chloroadenosine; NECA, 5'-N-ethylcarboxamidoadenosine; IBMX, Isobutyl-methyl-xanthine; R-PIA, R-N^6-Phenyl-isopropyladenosine; CHA, cyclohexyladenosine; CSV, calcium storage vesicle; GluNAc, N-acetyl glucosamine.

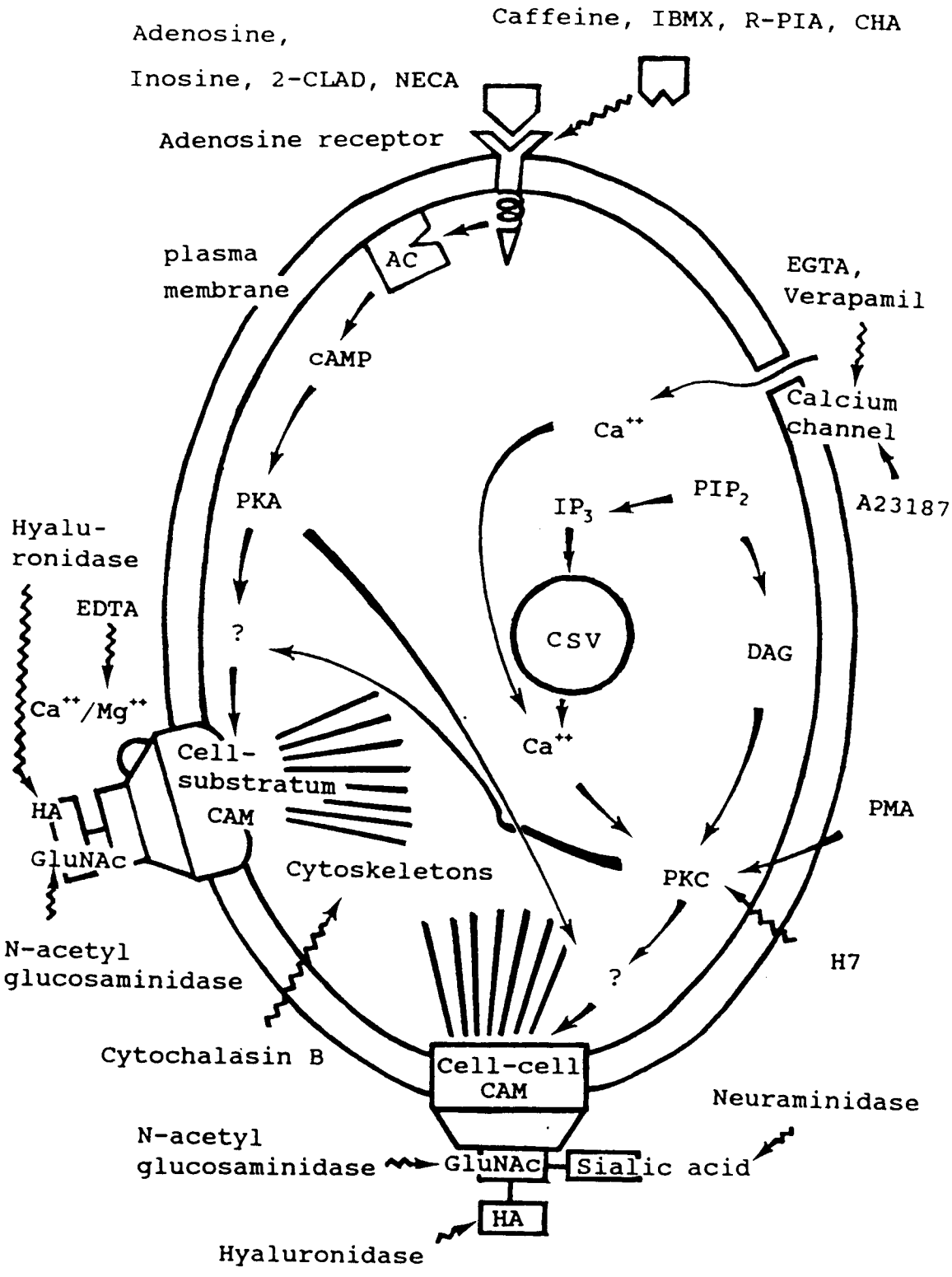

Adenosine,
Inosine, 2-CLAD, NECA
Caffeine, IBMX, R-PIA, CHA
Adenosine receptor
plasma membrane
AC
cAMP
PKA
?
Ca++/Mg++
EDTA
Hyalu-ronidase
HA
GluNAc
Cell-substratum
CAM
Cytoskeletons
N-acetyl glucosaminidase
Cytochalasin B
EGTA, Verapamil
Calcium channel
A23187
Ca++
IP3
PIP2
CSV
DAG
PKC
PMA
H7
Cell-cell CAM
Neuraminidase
N-acetyl glucosaminidase
GluNAc
Sialic acid
HA
Hyaluronidase

Figure VI-1

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