Studies on cell adhesion and activation

A thesis submitted to the University of Glasgow
for the degree of Doctor of Philosophy

By
Lakhdar Gasmi,
Department of Cell Biology, Glasgow University
November 1990

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgment

I am very grateful to Professor Adam Curtis for his supervision, helpful discussion throughout this project and for providing necessary facilities in his department.

Many thanks to Dr. Julian Dow for general help in the use of computing facilities, Dr. Hakim Djeha for reading the manuscript, Mr. Mike McGrath for general assistance in the laboratory, Mr.Andrew Hart for introducing me to photographic facilities,Mr.Gordon Campbell for introducing me to cell culture technics, and to all the other members of the Cell Biology Department for their general help and friendship.

I wish also to record my deepest thanks to my friends and family for their encouragement and the Algerian Government for financial support.

Contents:

Page number

List of abbreviations.

Ab	Antibody
ATP	Adenosine triphosphate
B10D2	Mouse pulmonary capillary endothelial cell line
BHK	Baby hamster kidney cells.
BPB	Bromophenacyl bromide
cAMP	Cyclic adenosine monophosphate
CAMs	Cell adhesion molecules
CD	Cluster of differentiation
CDS	Calcium dependent system
CIS	Calcium independent system
CoA	Coenzyme A
CS	Calf serum
CTL	Cytotoxic T-cells
DAG	Diacylglycerol
DLVO	Derjaguin-Landau-Verwey-Overbeek theory
DMSO	Dimethyl sulphoxide
E_A	Energy of attraction
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamine tetraacetic acid
Fab	Antigen binding fragment
FCS	Foetal calf serum
fMLP	(fMet.Leu.Phe) formyl-Methionine-Leucyl-Phenylalanine
G_1	Growth phase (Gap 1)
GAGs	Glycosaminoglycans
GD	Gangliosides
H	Hamaker constant
H-7	1-(5-isoquinolinesulfonyl)-2 methylpiperazinr.
HEPES	N-2 hydroxyethyl piperazine-N-2-ethane sulphonic acid
HS	Hepes saline
IL	Interleukin
IP_3	Inositoltrisphosphate
Kd	Kilodalton
L-CAM	Liver-cell adhesion molecule
LFA-1	Lymphocyte function associated antigen
LPS	Lipopolysaccharide
LTs	Leukotrienes
M	Molar

N-CAM	Neural-cell adhesion molecule
Ng-CAM	Neuro-glial-cell adhesion molecule
O_2^-	Superoxide anion
P	Probability
PEG	Polyethylene glycol
PGI_2	Prostacyclin
PGs	Prostaglandins
PIP_2	Phosphatidyl inositolbisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
R_f	Mobility relative to the front (TLC)
TC	Tissue culture
TLC	Thin layer chromatography
TPA	Tetradecanoyl phorbol acetate
TXs	Thromboxanes

list of figures

List of tables.

List of plates.

Summary.

The role of plasmalemmal lipids in the adhesion of Baby Hamster Kidney (BHK) fibroblasts to polystyrene tissue culture grade dishes in serum and serum-free medium was investigated using various fatty acids that differ in their hydrocarbon chain length and saturation. A number of biologically active agents which include arachidonic acid metabolites, as well as some of the factors which affect the calcium/protein kinase C pathway were also considered in the present study.

Fatty acids were the first to be tested. The saturated fatty acid derivative stearoyl-CoA (18: 0) had two different effects on BHK cell adhesion in serum-free Ham's F-10. Concentrations, ranged between 1.75 and 7 μM, enhanced the adhesion in almost all cases, while higher concentrations such as 14 and 28 μM reduced the adhesion of BHK cells to polystyrene tissue culture dishes by more than 50% of the control values.

BHK cells in the presence of long chain unsaturated fatty acids, (oleoyl-CoA (18:1), linoleoyl-CoA (18:2), linolenoyl-CoA (18:3), and arachidonoyl-CoA (18:4), responded in different ways depending on the type of the fatty acid added. When oleoyl-CoA was used at concentrations lower than 7 μM the adhesion was maintained at the control level or slightly enhanced. However,the use of concentrations higher than that reduced the attachment sharply.

Linoleoyl-CoA, linolenoyl-CoA and arachidonoyl-CoA generally maintained the adhesion at the control level, though linoleoyl-CoA enhanced the adhesion at fairly low concentrations (lower than 14 μM).

Both, stearoyl-CoA and oleoyl-CoA (1.75 μM and higher) decreased the spreading area of BHK cells by 30-50% of control samples in serum-free medium.

The above fatty acyl-CoAs did not however alter either the adhesion or the spreading of BHK fibroblasts in serum-containing medium.

In contrast to that, linoleic and arachidonic acid used as free acid form (unbound to the coenzyme A) at concentrations ranged between 2 and 20 μg/ml in combination with 1.25×10^{-5} M ATP and 5×10^{6} M

Coenzyme A, decreased significantly ($P<0.001$) the adhesion in both 3% serum and serum-free Ham's F-10 medium. A noticeable loss of viability was found at concentrations higher than 5µg/ml. The effect on cell viability was less pronounced in serum-containing medium.

Incorporation studies in serum-free conditions revealed that approximately 40% of the total labeled oleoyl-CoA was taken up by the cells in a period of 20 mins incubation. 21% of the total incorporated is present in the plasma membrane. This results suggest that the observed effect on cell adhesion might be due to changes in plasmalemmal lipids. Hence, the effect on the adhesion and spreading could be explained either in terms of the action of membrane electrodynamic forces or membrane fluidity. It should be noted however, that the total oleoyl-CoA incorporated was reduced by 43% when the experiment was carried out in serum-containing medium. This may explain the lack of effect of acyl-Coenzyme A on BHK cell adhesion as well as other functions in serum-containing medium.

The fatty acid metabolites, Prostacyclin (PGI_2), prostaglandin E_2, E_1 and leukotrienes B_4 (LTB_4) exerted different effects. At the time where 1.25 and 0.125 µM LTB_4 increased BHK and slightly endothelial cell-cell attachment and PGE_1 (1-50µM) enhanced BHK cell-polystyrene adhesion, prostacyclin and prostaglandin E_2 at concentrations up to 5 µg/ml did not have any clear effect on BHK cell-substratum adhesion.

Inhibitors of arachidonic acid release, Bromophenacyl Bromide and mepacrine (quinacrine), significantly reduced BHK fibroblast adhesion to polystyrene surfaces. There was an irreversible inhibition of adhesion. Viability tests revealed that approximately 95% of the cells were in a viable state.

Tests were also made on calcium and protein kinase C modulators. A diacylglycerol kinase inhibitor (R59022), reduced BHK fibroblast as well as endothelial cell adhesion to polystyrene tissue culture dishes in both serum and serum-free conditions. Endothelial cell adhesion in 3% serum Ham's F-10 was reduced by approximately 90% of the control samples when a concentration of 18.4 µg/ml was used. In contrast, a concentration of 30 µg/ml was needed to get the same reduction with BHK cells under the same conditions. The above concentration (30µg/ml) however, decreased BHK cell adhesion by 40%

of the control samples in serum-free conditions.

The adhesion of BHK 21 and B10 D2 endothelial cells treated with (10-40 μM) IP_3 was also reduced by 20-30% of the control in both 3% serum and in serum free Ham's F-10 medium. Such effect however, was reduced when cells were first preincubated for 60 mins at 37°C.

Using tritiated IP_3 , it was found that IP_3 binds to BHK cells in a concentration dependent manner. An approximate number of $27x10^3$ molecule bound per cell was detected using a concentration of 7.5 $x10^{-9}$ M radiolabelled IP_3.

The phorbol ester PMA at concentrations ranging between 1-4 μg/ml increased BHK cell adhesion gradually in 3% serum medium. No synergistic effect was found between PMA and ionomycin. Ionomycin did not have any effect on the adhesion in 3% serum Ham's F-10. An enhancement of cell adhesion however, was observed in calcium-free medium.

PMA at low concentration (10 ng/ml) in combination with PGE_1 did not alter BHK cell adhesion to polystyrene surface in 20 % serum-containing medium over an incubation time of 30 mins. Nevertheless, PGE_1 when added alone at concentrations ranging between 1-100 μM, BHK cell adhesion was increased. A 29% increase over control samples was observed at 10 μM PGE_1.

PMA at 10 ng/ml increased BHK cell spreading when added in mixture with PGE_1. The increase however, is less when PMA is added by itself. Cells treated with 10 or 100 μM PGE_1 for a period of 65 mins show a morphological change in their shape which included the appearance of process like growth and flattening. PMA (4μg/ml) and ionomycin (2-8μM) when added to the cells led also to a pronounced effect on cell shape. The cells started to elongate after 6 hours incubation and show a fibrillar morphology at longer incubation time. This shape change can be reversed by changing the medium.

This results suggest that calcium, protein kinase C and cAMP pathways might be controlling the adhesion and morphology of BHK fibroblast. Thus, these effects are explained in terms of activation phenomena.

The above results are fully discussed in the corresponding chapter.

Chapter I
Introduction

I. Introduction.

1.1. General.

Cell adhesion is defined as a process by which adjacent cells adhere to one another or to a non-cellular surfaces. It is a complex phenomenon of fundamental biological importance.

Warren Lewis, (1922), wrote that 'the adhesive quality of tissue cells is a subject of immense importance that up till that time had been almost entirely ignored'. He went on to point out that on this adhesiveness rests the integrity of tissues and organs and finally of the organism itself. He said " were the various types of cells to lose their stickness for one another and for the supporting extracellular white fibers, reticuli, etc., our bodies would at once disintegrate and flow off into the ground in a mixed stream of ectodermal, muscle, mesenchyme, endothelial, liver, pancreatic, and many other types of cells", (quoted from Trinkaus, 1969). In another word, there is no subject more fundamental than cell adhesion.

In order to proliferate (anchorage dependent cells), cells have to adhere to the substratum and spread into a characteristic morphology (McInnes *et al.*, 1987). Cellular adhesion is also important in morphogenesis and presumably in cell locomotion. A cell must adhere to a substratum strongly enough to gain the traction necessary for its movement, but not too strongly, else it would become stuck, or too weakly, so it would slip (Trinkaus, 1969). Thus, a cell must be able to adhere to the proper degree and deadhere constantly in a coordinate fashion.

Moreover, intercellular and cell matrix adhesion are critical during embryogenesis (see McClay and Ettensohn, 1987; Ekblom *et al.*, 1986 for review), in maintenance of tissue integrity and wound healing (Grinnell, 1984), in malignant behaviour (Weiss, 1984; Parish *et al.*, 1987) and in the immune system (Grant, 1973).

1.2. The biological significance of cell adhesion.

1.2.1. Embryogenesis. The body plan that arises at the end of early embryonic morphogenesis is mainly the result of temporally and locally coordinated events, that is proliferation, adhesion, migration, differentiation and death of cells (Duband and Thiery 1984).

Cell adhesion is widely believed to represent one of the most fundamental histogenetic processes for the development of multicellular organisms. In all solid tissues, cells adhere to each other and to the substratum. The adhesive events are thus instrumental both for embryonic development and for the maintenance of tissue architecture (Ekblom et al., 1986). The movement of cells, as exemplified by gastrulation and the migration of neural crest cells, are said to require specific adhesive interactions between cells and cell-extracellular matrix (integrins) (Thiery et al., 1985). For example, the folding of the neural plate into a neural tube may require the preferred adhesion of the neural tube cells with one another and a decrease in adhesion to surrounding mesodermal cells, while the migration of neural crest cells from the neural tube to specific areas of the developing embryo appears to involve adhesiveness to extracellular collagenous matrices (Weston, 1970).

Collagen, fibronectin and laminin are the major matrix glycoproteins identified so far and are found in association with a variety of different cellular phenotypes. In the early mouse embryo, laminin is the first matrix glycoprotein detected by immunofluorescence whereas fibronectin and type IV collagen are first found after implantation in the inner cell mass of the blastocyst, and interstitial procollagen is only detected several days later during differentiation of the mesoderm (Leivo et al., 1980).

Many investigators believe that cell adhesion molecules (CAMs) play a central role in morphogenesis by acting through adhesion as

regulators for other primary processes particularly morphogenetic movement. CAMs exercise their role as regulators of these processes by means of local cell surface modulation (undergo various forms), either by chemical modification or by changes in their prevalence on particular cells (Edelman, 1983). Several cell adhesion molecules have been now isolated and characterized (see later), among these are N-CAM and L-CAM which have been found in various vertebrate species (McClain and Edelman, 1982., Gallin et al., 1983). Their chemical properties and the nature of their tissue distribution suggest a key role for CAM's in early embryogenesis, in organogenesis and within mature tissues. N-CAM and L-CAM appear in specific set of pattern during early embryogenesis (used in organogenesis) and remain in adult tissue in distinct patterns that are related to their early distribution (Edelman, 1984a).

Particular attention has been also given to the presence of complex carbohydrates at the cell periphery and the role these macromolecules may play in mediating cell-cell interactions. In this respect, endogenous cell surface lectins have been considered as mediators of cell interaction in a variety of biological systems, including embryonic development (Monsigny et al., 1983, Rutherford and Cook 1981).

Numerous studies have underlined a possible involvement of glycosaminoglycans (GAG) in a variety of physiological processes during embryonic morphogenesis and tissue remodeling, and in cell differentiation (Gallagher et al., 1986). Because of their large polyanionic and hydrated domains, their situation in the extra- or pericellular matrix and their interaction with other extracellular matrix macromolecules or cellular components, GAGs, are ideal candidates for regulating interactions between the cells (Bouziges et al., 1989).

1.2.2. Repair and maintenance of tissue.

Cell adhesion plays a major role in the establishment and

maintenance of tissue and organs during the earliest stages of embryogenesis as well as in adult life (Edelman et al., 1983). It is now widely thought that the matrix provides attachment sites for the cells and that cell-matrix interactions are required for maintenance of the proper tissue histo-architecture. Experimental studies on tissue repair have shown that the basement membrane is important for the maintenance of tissue structure (Timpl and Dziadek 1986).

Platelet adhesion and aggregation (and their subsequent plug formation at the site of injury) play a major role in the control of haemostasis in the skin and arterial vessel wall (see MaCfarlane, 1976). Electron microscopic examination revealed that platelets filled gaps between endothelial cells (Tranzer and Baumgartner 1967) and formed a continuous layer on the subendothelial connective tissue at sites where endothelium had been removed (Baumgartner, et al., 1967).

1.2.3.Immune system. Leukocytes are found in the blood as non-adherent circulating cells, but their role in may pathological processes, is to leave the bloodstream and migrate into damaged or infected tissues. Their attachment to the endothelial lining the blood vessels, is therefore an important initial event in the acute inflammatory reaction (Hoover et al., 1980). Another critical step in the inflammatory response is the migration of circulating leukocytes from the blood stream to the injured extravascular tissue. Thus, leukocytes must cross the endothelium and the subendothelial basement membrane lining the vessel wall to reach the site of inflammation.

The migration of neutrophils through the endothelial junctions and surrounding perivascular structures has been well characterized morphologically using a variety of *in vitro* models (Shaw, 1980), but a little is known about the molecular mechanisms that govern this process.

Furie et al., (1987), demonstrated that neutrophil migration was a rapid event. They began adhering to the apical surface of the endothelium within two minutes following exposure to an fMet-Leu-Phe (fMLP) gradient, calcium ions were required for this initial adhesion.

Leukocyte adherence to vascular endothelial cells which precedes their emigration into extravascular tissue (Harlan, 1985 for review) is dependent on an interaction between the leukocyte receptors LFA-1 (Haskard et al, 1986) Mac-1 and/or P150,95 (Tevelde et al., 1987) and endothelial cell ligands.

In addition to the receptors for leukocyte cell-cell adhesive interactions, leukocytes also have multiple cell-matrix adhesion receptors. Evidence is emerging about such interactions of mature, active, motile lymphocytes with extracellular matrix components after penetration into extravascular tissue, and in matrix dependent maturation or movement of leukocyte within their tissue of origin (Hemler, 1988). Moreover, T-lymphocytes interact with target cell antigen and accessory cells, and this interaction is dependent on or augmented by several cell adhesion molecules such as the T-cell receptor-CD3 complex, LFA-1, LFA-3, CD2, CD4 and CD8 (Hemler, 1988).

Another type of cell adhesion involves interactions between specific lymphocyte homing receptors and high endothelial venule target structures, allowing recirculation to different lymphoid organs (Gallatin, et al., 1983, Hemler, 1988). Butcher et al., (1979), also demonstrated that mouse lymphocytes incubated on fresh frozen sections of mesenteric lymph nodes adhere specifically to the endothelium of high endothelial venules, specialised vessels through which lymphocytes normally enter the lymph node from the blood. B-lymphocytes and T-lymphocytes bind equally well *in vitro* and their binding demonstrates no regional preference

1.2.4. Malignancy. A vital part of the metastatic process is the attachment of circulating cancer cells to the vascular and perhaps

lymphatic endothelium. If the attachment does not take place metastasis can not occur. Cell adhesion is an essential part of active cell movement, unless a cell can stick to a substrate it can not crawl on it. Thus, cell adhesion is a vital component of the invasive phase of metastasis. Most generalized metastasis are blood or lymph borne, and during their circulation, cancer cells adhere to the vascular endothelium and those remaining in a viable state extravasate and form metastasis (Weiss, 1984). The interaction of tumour cells with the subendothelial matrix might also be expected to be a necessary step for extravasation (Kramer & Nicolson, 1979).

The nature of the adhesive molecules or forces in cell contact phenomena relevant to tumour cells is poorly understood.

It was found that metastatic cells bind preferentially to basement membrane type IV collagen using laminin as an attachment protein (Terranova et al., 1986). In animal studies, it has been found that antibodies to laminin (Terranova et al., 1982), as well as fragments of laminin (Barsky et al., 1984), and the YIGSR synthetic peptide (Graf et al., 1987), which bind to laminin receptor, reduce the formation of metastasis when injected together with the cells.

Cheresh et al., (1984), presented data indicating that disialogangliosides GD_2 and/or GD_3 are predominant gangliosides synthesized by two human melanoma cell lines. They showed also that these gangliosides are present in the membrane and in focal-adhesion plaques, implicating them as molecules that may play an important role in the adhesion of melanoma cells to substrate.

In later paper, Cheresh and his coworkers demonstrated that monoclonal antibodies directed specifically to the carbohydrate moiety of GD_2 and GD_3 inhibit melanoma and neuroblastoma cell attachment to various substrate adhesive proteins, including collagen, vitronectin, fibronectin and laminin (Cheresh et al., 1986). They suggest that cell-substratum interactions may depend in

part on the electrostatic environment provided by terminal sialic acid residues of cell surface gangliosides and possibly other anionic glycoconjugates.

A role for gangliosides in cellular adhesion has also been suggested by other studies that demonstrated increased adhesive properties of cells whose membrane were enriched with exogenous gangliosides (Yogeeswaran, 1981).

Possible abnormalities in cell-cell contact may be a primary cause of abnormal cell proliferation. Abnormalities in cell adhesion may also be related to metastasis of cancer cells. A number of studies have conducted comparing the adhesiveness of normal and transformed cells. One of the earliest attempts was the work done in the early forties by Coman, (1944). He measured the strength of adhesion between cancer cells and found that less distractive force was required to separate a limited number of pairs of carcinoma cells in comparison with the corresponding normal cells. Therefore, he proposed that the malignant state is characterised by a decrease in the adhesion of malignant cells than normal cells. Other studies have shown that the surface potential of tumourous cells was higher than in normal cell type (Ambrose et al., 1956, Forrester et al., 1964). This might support the view that the electrostatic force of repulsion is controlling cell adhesion.

1.3. Mechanisms of cell adhesion.

The mechanism of cell adhesion is still one of the unsolved problems in cellular and developmental biology. The inability to find a unified mechanism for cell adhesion is due to the complexity of cell surface components as well as the physiological environment of cells in the organism.

It is well documented that most cells adhere to most surfaces, whether the surfaces are those of other cells or non-living materials (see bellow).

For instance, amoebae of *Dictyostelium discoideum* attach and spread on hydrophobic methylated glass and hydrophilic clean glass as well as acid treated polystyrene and polylysine adsorbed on glass (Gingell and Vince 1982). Human red blood cells stick to glass in physiological saline (George, et al., 1971), and adhere to protein free liquid hydrocarbon interface and clean glass (Todd and Gingell 1980; Gingell and Todd 1980). Tissue cells in culture adhere to polystyrene or glass in the presence or absence of added serum proteins (Rabinovitch and De-Stefano, 1973; Harris, 1973; Grinnell, 1976), and fibroblasts adhere to glass, polystyrene, hydroxylated polystyrene, and to paraffin wax (Curtis, 1964; Maroudas, 1973,77; Martin & Rubin, 1974, Curtis et al., 1983).

On the other hand, there is a clear ability of cells to adhere and spread very well on surfaces coated with different proteins.

Grinnell and Feld (1979), reported that fibroblasts have an absolute requirement for fibronectin for attachment and spreading on polystyrene surfaces. This however, was later disproved by Curtis and McMurray 1986, who showed that fibroblast adhesion can take place in the entire absence of fibronectin. Cells from *Xenopus laevis* gastrulae showed rapid adhesion and active movement on fibronectin or laminin-coated surfaces (Nakatsuji, 1986). Bovine corneal endothelial cells and BHK-21 cells attach to fibronectin as well as to vitronectin coated substratum (Underwood and Bennett 1989). They reported also that vitronectin is the active attachment/spreading factor in serum containing medium. Norris et al., (1990), showed that endothelial cells attach to and spread on collagen I and IV in serum medium without any requirement to serum fibronectin or vitronectin.

It should be noted however, that the studies carried out on cell adhesion in serum-containing media is very complicated by the fact that under this conditions there are nonadhesive proteins that would adsorb preferentially to the surfaces used for the adhesion assay and compete with other adhesive proteins (Curtis and

Forrester 1984). Proteins also adsorb to both cellular and noncellular surfaces when these surfaces are placed in protein solution. This adsorbtion could affect the properties of cell surface as well as substratum to which cells adhere. The use of serum-containing media or surfaces coated with serum proteins was shown to make the cell adhesion dependent on the presence of serum. Nevertheless no such dependence was seen when cells did not come in contact with serum-containing media even for transient period (see Curtis and McMurray 1986).

Because of the clear ability of cells to adhere to diverse clean or coated surfaces in serum or serum-free media, it is worth looking for a mechanism responsible for a generalized adhesiveness.

A number of theories has been introduced in purpose to explain the mechanism of cell adhesion. These theories can be divided into two major categories, the physico-chemical and the molecular theory.

1.3.1. The physico-chemical theory.

This is based on general cell surface properties, which include charge, fluidity and hydrophobicity. Some simple physical models of particle interaction are relevant to cell surface behaviour in adhesion. Two similarly charged spherical particles generate a certain electrostatic potential energy of repulsion, a force that varies with distance, the closer the greater. Such particles will adhere to each other when they overcome the repulsive force by the action of forces of attraction.

If we consider cells as charged spheres that behave like flocculating colloidal particles, then the repulsive and attractive forces that govern particle flocculation might also apply to cell adhesion.

The main systems which may act to produce adhesion were reviewed by Curtis 1967, 1973; Gingell & Vince 1980, from which the following in brief are:

- Bridging systems.
- Electrostatic interactions.
- Interactions involving long-range forces.

1.3.1-1. Bridging systems. The bridging agent is a molecule which combines in some ways with both surfaces and links them together. Two main classes of bridging mechanism have been recognized:

i. Each end of the macromolecule may adsorb to the surface of each particle if the length of the molecule is greater than twice the range of the electrostatic forces of repulsion of each surface.

ii. Polyvalent ions of relatively small size and that bear a charge opposite to that of the surface, bind to charged grouping on either surfaces to provide the bridging materials if the surfaces are close enough.

1.3.1-2. Electrostatic forces of attraction. These forces are the consequence of the presence of two surfaces that have mainly an opposite sign of charge or a mosaic of charge of either sign so that areas of opposite signs face each other. Bierman (1955), have suggested that surfaces of the same sign of charge, but differing potential, may show very short-range attractive interactions.

1.3.1-3. Interaction involving long range forces (DLVO Theory). The DLVO theory was introduced by Derjagin & Landau, (1941) and Verwey & Overbeek (1948) to explain lyophobic colloid particle interactions. It is well known that cells have a net negative charge at their surfaces, and therefore, resemble lyophobic colloid particles. Thus, it was not surprising that the theory of lyophobic colloid interaction was applied to study cell interaction (Curtis 1960, 1967; Weiss & Harlos, 1972).

The DLVO theory considers adhesion to be determined by the balance of electrostatic forces of repulsion which tend to keep the charged particles apart, and the London-vander Waals (electro-

dynamic) forces of attraction which tend to bring them together.

i. The electrostatic (like-charge) force of repulsion. This force develops between surfaces of like sign and charge density. Under physiological conditions, a repulsive force should always exist between cells. However, this may not be sufficient to overcome some force of attraction.

ii. The Van der Waals-type force of attraction. It is an electrodynamic force in its origin (Dolowy, 1980). It arises from the fluctuating dipoles due to the atomic and molecular vibration. These fluctuations give rise to electromagnetic interactions with the dipoles of neighboring atoms and molecules, such that, they are polarized and a force of attraction develops. This attraction only develops between atoms which have similar fluctuation frequencies (Curtis, 1973).

The attractive and repulsive forces obey different force laws, and each of them vary with distance but in different ways. While the attractive force dominates at larger separations of interacting surfaces, the electrostatic repulsive force is stronger at short distances (Dolowy, 1980). As a result, the total energy of interaction between two surfaces will also vary with distance. Therefore, the energy barrier to contact lies between two regions of attraction, a short range (primary minimum) and a long range or secondary minimum. Anything that reduce repulsive forces, particularly in the region of the primary minimum where attractive forces are high, should promote close contact. The existence of secondary minimum might be of relevance for cell interaction (Curtis, 1967).

The diagnostic features of primary minimum adhesion are:

1. No gap is found between the interacting cells.

2. The adhesion is irreversible.

3. The formation of the adhesion will depend on the relative magnitude of electrostatic and London forces.

4. Such adhesion will show no specificity, for any two particles

having approximately the same dielectric constant immersed in a medium of very different dielectric constant will show adhesive behaviour if the electrostatic forces of repulsion are not too great.

Secondary minimum adhesion will be characterized by:

1. The presence of a gap between the interacting particles.

2. The adhesions are reversible, because they are of relatively low energy.

3. The narrower the gap the stronger the adhesion is.

4. Limited specificity.

5. Such adhesion will only be found between large particles (> 500 Å radius).

6. Provision of the potential energy barrier to the formation of the primary minimum.

7. Increasing shear rate decreases such adhesion.

1.3.1-4. Evidence for the physico-chemical theory.

1. Bridging mechanisms.

Evidence in favor of the involvement of bridging mechanisms in cell adhesion arises from:

i. Demonstration that adhesion requires a process of synthesis (Moscona, 1961; Roth, 1968). Two factors have been widely interpreted as evidence for synthesis of a bridging agent.

a. The effects of changes of temperature on cell adhesion. The decreased adhesion of trypsinised cells at low temperature has been interpreted in most instance as dependence upon the synthesis of a binding macro-molecule (Moscona, 1961; Roth, 1968; Edwards and Campbell 1971). An alternative explanation is that certain methods for the preparation of cell suspensions damage the cell surface and that a repair synthesis is required before the adhesion occur (Curtis, 1973).

b. The effect of inhibitors of protein synthesis on the formation of adhesion. Many workers believe that the inhibition of

adhesion is a result of the inhibition of protein synthesis (Moscona, and Moscona, 1966; Richmond et al., 1968). However, Dunn et al., (1970), established that puromycin depressed the carbohydrate metabolism of trypsin-dissociated fibroblastic cells in addition to blocking protein synthesis. The pattern of inhibition of aggregation was consistent with the gradual depression of the metabolism rather than the rapid effect on protein synthesis. Weiss and Chang (1973), presented a data showing that metabolic inhibitors cyclohexamide, puromycin and actinomycin D, increased the rate of adhesion of Ehrlich ascites tumour cells to coverslips and plastic surfaces over a period of up to two hours *in vitro*. They explained this as a decrease in the production of hypothetical anti-adhesion factor.

ii. The isolation or visualization of substances that might be the bridging materials (Moscona, 1962; Lilien and Moscona, 1967; Takeichi; 1971; Kondo and Sakai, 1971).

iii. The demonstration that adhesion is specific (Humphreys, 1963; Roth et al., 1971).

iv. Indication that techniques for dispersing tissues act by lysing or de-sorbing bridging agents between the cells so that they lose their adhesiveness (Moscona, 1963; Edwards and Campbell 1971).

2. Electrostatic and electrodynamic forces.

i. General. To demonstrate an action of electrostatic forces of repulsion in cell adhesion, the prime requirement is to demonstrate that raising surface potential of a cell is correlated with a diminution in adhesiveness, or that the application of ionic conditions that would alter the range and intensity of electrostatic forces would affect adhesion in coordinate manner.

Low pH, high ionic strength and the presence of divalent cations all favour adhesion in most instances (Curtis, 1967).

As early as 1936, Dan measured the adhesiveness of various echinoderm eggs to glass and the zeta potential of the eggs for a

variety of ionic conditions. He found that as surface potential increased, adhesiveness decreased. Adhesion was measured by observing the resistance of the cells to their detachment under gravity from a glass surface. The electrophoretic mobility of hamster kidney cells and their stilboestrol induced tumourous form was measured, and it is found that the surface potential was higher in the tumourous form than in the normal cell type (Ambrose et al., 1956). The tumourous form was supposed to be less adhesive.

Curtis, (1964), observed that embryonic chick cells in culture tended to de-adhere from their adhesions to glass under conditions where electrostatic repulsive forces would be of greater magnitude than in normal tissue culture conditions.

Forrester et al., (1964), examined the malignant behaviour of transformed hamster kidney cell line produced by viral infection and showed that increased surface potential was associated with the development of malignancy as judged by their behaviour in tissue culture.

In a simple experiment on the aggregation of human red blood cells (RBC) treated with glutaraldehyde, it was found that RBC remain single after 3 hours oscillatory shaking in 150 mM saline at a series of pH values (Gingell and Vince 1980). As the pH falls at constant ionic strength, the surface sialic acid carboxyls associate, the electrostatic force of repulsion falls and the cells aggregate.

Margolis et al., (1978), using films of phospholipids and glycerides on glass, observed that fibroblast adhesion and spreading in the presence of serum proteins is reduced on films whose phase-transition temperature is below 37°C but takes place on films which are in gel state at 37°C. They suggest that the increased spreading on films in the gel state at 37°C may indicate increased adhesion due to electrostatic interaction with phospholipids' head group.

ii. Modification of cell surface.

The mechanisms of cell adhesion are controlled by the chemistry of the cell surface. Thus, modification of cell surface charged groups by enzymic treatment, as well as by other chemical procedures, is means of testing the importance of electrostatic forces in both cell-cell and cell-substratum adhesion.

Several authors (Kemp, 1970; Roseman, 1970) examined the possible role that sialic acid residues (present on the cell surface) might play in cell interaction. These derivatives of neuraminic acid possess carboxyl groups that are fully ionized at physiological pH, and usually are found at the ends of oligosaccharide chains of plasma membrane glycoproteins and glycolipids (see Curtis, (1967) and Weiss, (1973) for review). The significance of sialic acid residues can be tested using specific enzymes, neuraminidases, to remove some of these residues from the cell surface. This results in a lowering of their surface charge density (Cook et al., 1961; Kemp, 1970).

Thus, if electrostatic forces act in adhesion, it would be expected that such treatment would increase the adhesiveness of the cells.

Kemp (1970) reported, using optical density measurement to fellow the rate of aggregation of an agitated cell suspension, that the aggregation of trypsinised embryonic chick muscle cells was slightly greater in controls than in cells that aggregate in the presence of the enzyme. Kolodny (1972), also, found that neuraminidase treatment did not affect the rate of attachment of trypsinised cells to the substrate. They argued that these results appear to be in contrast to that expected if electrostatic forces act.

In contrast to these, Allen and Cook (1970) reported that neuraminidase treatment of macrophages increased the percentage of attached bacterial cells. Similarly, Vickers and Edwards (1972), measured the aggregation rate and extent of trypsinised BHK cells

treated with neuraminidase. They found that neuraminate was released from the cell surface and that the adhesiveness of the cells increased.

Lloyd and Cook (1974), showed that the exposure of briefly trypsinised malignant dermal fibroblasts to neuraminidase increased their aggregation as measured by gyratory shaker/electronic particle counting technique.

Hoover, (1978) showed that the preincubation of either PMN leukocytes or endothelium with neuraminidase increased the interaction of PMN leukocytes with endothelium cells. He found that divalent cations were necessary for this adhesion, suggesting that divalent cations can also function in reducing charge density by binding to the negative sites on the surface (Collins, 1966).

Dennis et al., (1982), reported that sialic acid reduces attachment of metastatic tumour cells to collagen type IV and fibronectin, in that, neuraminidase treatment of wheat germ agglutinin-sensitive metastatic tumour cells enhanced their attachment to collagen type IV and fibronectin, but not to laminin.

The effect of changing plasmalemmal sialic acid on cell adhesion is best explained in terms of DLVO theory, in that removing sialic acid removes charge, and thus forces of repulsion is more plausible.

iii. Modification of substrate.

The chemistry of the substrate to which cells attach is also an important element in determining cell adhesion. Therefore, changing the surface chemistry might provide a good evidence for the physico-chemical theory.

Polystyrene dishes for instance have been used for cell culture since about 1965. Many cell types adhere to and move on the surface of such materials and present a morphology similar to that seen when the cells are grown on glass. However, it has long been known that the polystyrene surface must be subjected to a surface treatment to make it suitable for cell attachment.

Curtis et al., 1983, investigated the treatment required to render polystyrene surface suitable for cell adhesion. Using x-ray photoelectron spectroscopy to measure carboxyl-, hydroxyl- and sulphur-containing groups, they showed that sulphuric acid treatment of polystyrene surface produced an insignificant number of sulphonic acid groups. They demonstrated also that this technique as well as other oxidation procedures rendered surfaces suitable for cell culture and generated high surface density of hydroxyl groups. Blocking these hydroxyl groups inhibited the adhesion of BHK and leukocytes but no effect was observed when carboxyl groups were blocked. These results applied to adhesion in the presence and absence of serum.

In later paper, Curtis and his colleagues (1986) demonstrated also that hydroxyl groups were required for cell adhesion, though at very high surface densities of these groups, the adhesion is diminished. Their work showed also that BHK cells possess the ability to modify appropriate surface by oxidation. They suggest that such oxidation may explain some of the conditioning effect that cells have upon substrates and raise questions about the extent to which the cells can modify their lipids and those of their neighbours by producing oxidation products.

Owens et al., (1988), used red blood cells and *Dictyostelium discoideum* and demonstrated that alcoholic hydroxyl groups in a two dimensional array, provide a surface that is strongly adhesive for cells.

Action of long range forces in cell adhesion comes also from the following lines of investigation.

a. The measurements of the energy or forces of adhesion for cell to cell and cell to substratum.

b. The morphology of the cell contact.

c. The effect of changes in the di-electric constant of the medium or plasmalemma.

The DLVO theory predicts that cell adhesion is the consequence

of balance between the electrostatic force of repulsion and van der Waals force of attraction.

In most instances the van der Waals force will operate in the presence of an electrostatic force of repulsion because of the charged groups on the surfaces. Therefore, experimental investigation of the possibility of the action of london force necessarily implicates the action of the electrostatic forces in nearly all cases. It is necessary therefore to find an experimental treatment which alters the london force without altering electrostatic forces.

a. Measurement of adhesive energies. There are two ways of describing the van der Waals force. The first use *H*, the Hamaker constant as mathematical representative of all interactions between all molecules of adjacent surfaces by a single form. The second way is based on the fact that all the electron fluctuations of the molecule influence the electromagnetic field and thus can be detected spectroscopically, from which the force can be calculated.

The van der Waals energy per unit area of two interacting flat surfaces is given by the following equation:

$E_A = -H / 12\pi d^2$

in which E_A: is the attractive van der Waals type energy per unit area.

H: Is the Hamaker constant.

d: Is the distance or the thickness.

Many workers in this field have obtained values for the Hamaker constant ranging between (10^{-23} - 10^{-20} Joules) for a variety of lipids and cells (Wilkins et al., 1962; Bangham et al., 1967; Curtis 1969; Parsegian and Gingell 1980). The above values were based on the assumption that the van der Waals forces were the main source of attraction in the system under investigation. Theoritical and experimental calculations have been made in order to measure forces and energies of cell adhesion.

Brooks et al., (1967) calculated a secondary minimum energy of -20 *KT* for cells of radius equal to 5μm. They found also a shear force of about $1.5x10^{-9}$ N per cell required for separating cells. Weiss (1968) obtained a value for the force of adhesion in the secondary minimum of $6x10^{-11}$ N for an adhesion to glass. Curtis (1969) using aggregation studies also obtained values for the energy of adhesion for a variety of cells ranging from 10^{-9} to 10^{-7} and 10^{-5} and 10^{-4} J m^{-2}. He suggested that this values correspond to secondary and primary minimum respectively.

Parsegian and Gingell (1972), calculated a secondary minimum adhesion at a separation of 40-80 Å with a depth of $1x10^{-11}$ to $1x10^{-10}$ J m^{-2} $x10^{-4}$. Gingell et al., (1977) presented an experimental evidence that adhesion of red cells to an oil-water interface is mediated by an attractive force acting at a distance apparently exceeding 100 nm. They calculated the gravitational force of an average red cell, and found it to be 8 $x10^{-14}$ N. They argued that this is the size of the calculated electrostatic repulsion in 0.3 mM NaCl for a cell to interface separation of about 240 nm in which an average cell is about to lose its adhesion. They suggest that a cell sedimenting down onto the oil/water interface under gravity alone can not approach closer than 240 nm. Thus without an attractive force, cells would balance at 240 nm. Parsegian and Gingell (1980) showed that the electrostatic force of repulsion sufficient to prevent adhesion of gluteraldehyde-fixed red blood cells to metal/saline and liquid hexadecane/saline interface are remarkably similar and that a force-balance condition is predicted at cell-substratum separation of ~100nm in 0.4 mM NaCl as found also by interferometry examination (see Gingell and Todd 1980).

b. The morphology of the cell contact. Curtis (1962; 1967), suggested that the various type of cell contacts (junctions) between interacting cells represent a secondary and primary

minimum. The zonula adherens (belt desmosome) represent a secondary minimum situation, while the zonula occludens (tight junctions) in which the two plasmalemma appear to come or nearly come into molecular contact, is a primary minimum situation. The gap junction can be regarded as development from the primary minimum adhesion in which the contacting surfaces have undergone a structural rearrangement into a single hexagonal structure. Interference reflection microscopy (IRM) (Curtis, 1964) appeared to confirm the morphology in life. Curtis, (1964) using an optical interference method, was able to measure the thickness of the gap between a cell and the glass surface to which it was adhering.

c. The effect of change in di-electric constant. In order that a long range attraction develop between surfaces, the di-electric constant of the surfaces as well as of immersion medium should be different (Curtis, 1973). Therefore, the modification of the dielectric constant of surfaces or immersion medium may provide a strong test for the action of long range attractive forces. Jones, 1974, investigated the effect of increasing the dielectric constant of the emersion medium on cell adhesion. He used different aqueous solutions, in particular glycine and diglycine, and observed that there was a steady increase in the adhesive interaction of the cells with increasing the dielectric constant of the medium.

The di-electric constant of the cell surface arises from the nature of its components. Therefore, the hydrocarbon chain length and degree of unsaturation of the fatty-acid component of phosphatidyl- and sphingo-compounds may play an important role in determining the value for di-electric constant for cell surface (see below for details).

3. Membrane lipids and cell adhesion.

Another approach to adhesion has been introduced by Curtis et al., (1975a,b,c) and Schaeffer and Curtis (1977). This approach

concentrates on the relation between plasma membrane lipids modification and cell adhesion.

Over 60 years ago, Gorter and Grendel proposed that membrane lipids are organized into a sheet two molecules thick, with polar head groups facing outwards and nonpolar tails inwards. After, Singer and Nicolson, (1972) and Curtis, (1972) proposed the mosaic fluid model which regards biological membranes as two dimensional solutions of oriented globular proteins in fluid lipid bilayer phase. Depending on the origin of the cell, lipids contribute from 20% to 80% of the weight of the membrane (Guidotti, 1972). Plasma membranes consist of lipids and proteins both of which may be glycosylated. These components are combined into a three dimensional supramolecular organization, held together mainly by noncovalent bonds. The lipids and proteins interact and introduce principles of regulation and cooperation.

Membrane lipids are divided into three classes:

i. Phospholipids: They form a dominant part of all biological membrane and they are strikingly fitted for this by the polar-nonpolar asymmetry of their molecules. Phospholipids represent two major subclasses:

a. Phosphoglycerides: Alcohol esters, their phosphate is bound to any of choline, serine, ethanolamine or inositol, and their backbone is glycerol.

b. Sphingomylins: alcohol esters also, their head group is choline, and the backbone is sphingosin.

ii. Neutral lipids. are represented by cholesterol which interacts with phospholipids molecules to decrease the motion of the fatty acid chains and hence increases the bilayer viscosity which leads to a decrease in permeability (Alberts et al., 1983).

iii. Glycolipids: Lipids whose head group comprises one or more glycosyl molecules such as cerebrosides, gangliosides, etc.

The cell surface is generally negatively charged, by sialic acid residues of glycoproteins, carboxyl groups of proteins and phosphate (Seaman, 1973). Many cellular functions are known to be

controlled by the cell surface. The membrane as a selective barrier is controlling the exchange of compounds between cells or cells and the external milieu (Hoover, 1978). Therefore any perturbation of this organelle, leads to a change in proteins, lipids and/or carbohydrate function which in turn affect many cellular activities.

Membrane lipids are known to be in a dynamic state with significant rate of turnover of the whole molecules and parts of individual phospholipid molecules (White and Tucker, 1969). Changes in membrane phospholipids may therefore serve a major regulatory function.

Selective interaction between lipids and proteins may control protein conformation (Fourcans & Jain, 1974., Sandermann, 1978) and thus determines enzyme activities, antigenic and receptor expressions, and imposes allosteric regulation (Emmelot, 1977). For example, many membrane enzymes require phospholipids for activity (Chapman, 1973), and it is lost if the fatty acid residues are changed from unsaturated to saturated or if a single acyl residue is removed, resulting in formation of lysophosphatide (Rothfield and Romeo, 1971).

Several lines of evidence indicate that adenylate cyclase is dependent upon membrane lipids for basal and hormone-stimulated activities (Engelhard et al., 1976).

Houslay et al., (1976) have used the technique of lipids substitution and fusion to introduce up to 60% of synthetic phosphatidylcholine into the lipid bilayer associated with the membrane (rat liver plasma membrane), and demonstrated that the coupling of the receptor to the catalytic unit changes adenylate cyclase activity in a manner consistent with the phase properties of the exogenous phosphatidylcholine.

Mitochondrial β-hydroxy butyrate dehydrogenase is only active in the presence of the zwitterionic phosphatidyl choline (Fleischer et al., 1974). While Na, K-ATPase is particularly active in the

presence of negatively charged lipids (Depont et al., 1978).

Several possible mechanisms can account for the effect of lipids on enzyme activity.

a.The lipids can activate the substrate. The lipids interact physically with the substrate, thereby converting it into an active participant in the reaction.

b.The lipids can directly activate the enzyme, perhaps by inducing a conformational change in the protein.

c.The lipid can act as an organizer in multienzyme systems, directing the sequential arrangement of enzyme proteins within the membrane such as the electron transport system (Rothfield and Romeo, 1971).

Changes in membrane phospholipids, such as stimulating lysophospholipid accumulation, or fatty acid incorporation or changing the polar head group was found to alter cell-cell and cell-substratum adhesion (Curtis et al., 1975 a,b,c. & Hoover et al., 1977).

Curtis et al.,1975c, suggested that the effect might be due to changes in intermembrane Vander waals forces (DLVO. theory). They indicated that conditions that lead to lysophosphatide compounds accumulation in the plasma membrane reduced adhesion, while conditions which might lead to its reacylation may increase adhesion (Curtis et al.,1975a). They showed also that addition of lysophosphatidyl compounds or phospholipase A_2 (PLA_2) decreased the adhesion of neural cells of the retina.

Fischer et al., (1967), reported that red blood cells in which lysocompounds have accumulated will not clump (aggregate). They described also, the existence of a system in the red cell surface for the reacylation of lysocompounds.

Curtis et al., (1975c), showed that stimulation of such system could be used to reverse the effect of lysophospholipid accumulation in the plasmalemma. They demonstrated that adhesion of

retina cells after 20 minutes incubation with fatty acids, ATP and coenzyme A (10 μg/ml, $1.25x10^{-5}$M, $5x10^{-6}$M respectively), is greater the longer the chain length of the fatty acid used or the greater the saturation of the molecules. They explained this effect in terms of changes in membrane fluidity, or changes in intermembrane van der Waal force.

Hoover et al., (1977), showed that BHK cells can incorporate exogenous fatty acids which can then cause changes in growth and in both cell-cell and cell-substratum adhesion. They indicated that, long chain unsaturated fatty acids (arachidonic, linolenic and linoleic acids) decreased the adhesion, while long chain saturated acids (stearic, palmitic) had a little or no effect on adhesion.

Williams et al., (1974), found that mouse L.M cells cultured in either 8μg/ml linoleic acid-Tween ester or 16μg/ml linolenic acid-Tween ester, incorporated large amounts of the polyenoic acids into the cellular lipids and exhibited a morphological abnormality and loss of viability.

Weeks, (1976), showed that the incorporation of poly-unsaturated fatty acids into the slime mold *Dictyostelium discoideum*, impairs cell-cell contacts which in turn affects differentiation.

The mechanism by which lipids affect these cellular functions which include adhesion, growth, differentiation and viability is still unclear. However, Hoover, (1978) suggested two possible mechanisms:

1. That there is a direct change in the lipid composition of the cell surface which alters the fluidity of the membrane. This was demonstrated by Curtis et al., (1975 b & c) and Schaeffer and Curtis (1977).

2. That lipids are acting intracellularly through the synthesis of fatty acid metabolites.

1.3.2 Molecular theories of cell adhesion.

In recent years much research in the field of cellular adhesion has

been devoted to the identification and study of glycoproteins which are thought to be cell adhesion molecules. These, include those molecules mediating cell-cell adhesion as well as those of the extracellular matrix which might have their appropriate receptors on the cell surface, and may play a major role in cell-substratum adhesion.

1.3.2-1. Cell-cell adhesion. It is a fundamental process in the construction of multicellular animals, and a number of studies have been undertaken to elucidate its molecular mechanisms. cell-cell adhesion are thought to be initiated by cell adhesion molecules (CAMs) and intercellular membrane junctions.

1. Cell adhesion molecules (CAMs).
CAMs are a class of integral glycoproteins that are believed to initiate and maintain cell-cell adhesion (Edelman, 1984b, 1985).
The original identification of most cell adhesion molecules was based on the observation that monovalent antibodies (Fabs) directed against CAMs, interfere with cell-cell adhesion as measured in *in vitro* assays (Beug et al., 1970; 1973). Such an approach can identify candidates for cell adhesion molecules, but antibody inhibition of adhesion alone is not a proof of adhesive function (McClay and Ettensohn, 1987). The binding of antibodies to the cell surface may stericaly block access to molecules other than the specific antigen, or may affect cell adhesion indirectly by perturbing the function of a regulatory molecule that has global effects on cell adhesion (Gerisch, 1986). However, it is of general interest to give a brief history of the isolation and characterization of CAM´s.

i. Isolation. Cell adhesion molecules were first definitively identified by means of immunologically based adhesion assays in which specific antibodies capable of blocking cell adhesion (*in*

vitro) were used to purify cell surface molecules as putative CAMs (Brackenbury et al., 1977, Thiery et al., 1977, Gallin et al., 1983, etc.).

Using this assay, it was possible to identify on chick embryo neurons a neural cell adhesion molecule (N-CAM) (Brackenbury et al., 1977, Thiery et al., 1977). The same procedure allowed the identification of two other molecules in the chick. Liver cell adhesion molecule (L-CAM) originally isolated from embryonic liver cells (Gallin et al., 1983), and neuro-glial cell adhesion molecule (Ng-CAM), isolated from neurons and known to mediate neurons and glial cell adhesion (Grumet and Edelman, 1984).

Beside the above cell adhesion molecules, others have been found in mammals. These include, the proteins BSP-2 and D-2 (Goridis et al., 1984; Jorgensen et al., 1980), that are specific for neurons and share similarities with N-CAM. Uvomorulin (Hyafil et al., 1980) and E-cadherin (Yoshida-Noro, 1984) perform similar function to L-CAM. A protein L-1 have been found identical to Ng-CAM (Lindner et al., 1983).

On the bases of metal requirement the known CAMs can be classified into two groups.

Calcium dependent CAMs which include L-CAM (Gallin et al., 1983), uvomorulin (Hyafil et al., 1981), E-cadherin (Yoshida-Noro, 1984), require calcium for adhesion and are protected against proteolysis in its presence (Takeichi et al., 1983).

Calcium independent CAMs include N-CAM (Hoffman et al., 1982) and Ng-CAM (Grumet and Edelman, 1984).

ii. The structure and biochemistry of CAMs.

1. N-CAM. It is an integral membrane protein of 160 kilodalton (Kd). It consists of three related polypeptide chains (Hoffman et al., 1982; Hansen et al., 1985), each of which has three domains that are very susceptible to enzyme proteolysis. An N-terminal binding domain, and a cell associated or cytoplasmic domain (Hoffman et al., 1982; Cunningham et al., 1983).

2. L-CAM. It is a calcium-dependent cell adhesion molecule found in very early vertebrate embryos and on liver and other epithelial cells in adults (Gallin et al., 1985).

Studies by Gallin and his co-workers (1983), indicate that L-CAM is an acidic glycoprotein that appears on the cell surface as an integral protein of at least 124 Kd. Trypsin, in the presence of calcium, releases L-CAM from membrane as a 81 Kd polypeptide, but in the absence of calcium, L-CAM appears to be degraded by trypsin, and only relatively small amounts of a 40 Kd fragment are detected (Gallin et al., 1983). L-CAM consists of an amino-terminal binding region and a cell-associated region and contains also several sites susceptible to proteolytic cleavage in the absence of calcium ions (Cunningham et al., 1984).

3. Ng-CAM. It is a glycoprotein that mediates the heterotypic adhesion between neurons as well as between neuronal membrane and glial cells (Grumet et al., 1984a). Ng-CAM was localized by specific antibodies on neurons but not on glia, and double-staining methods, showed that individual neurons contained both Ng-CAM and N-CAM (Grumet et al., 1984a).

Three polypeptide components of chicken Ng-CAM (200, 135 and 80 Kd) have been isolated. Using specific antisera against each component, the 135 and 80 Kd components were found to cross react antigenically with the 200 Kd component but not with each other. This suggests that the 135 Kd and 80 Kd are structurally related to different regions of the 200 Kd molecule (Grumet et al., 1984b).

2. Intercellular membrane junctions.

The three known junctions are gap junctions, tight junctions and desmosome. Their main functions are the formation of specialised cell-connections, cell communication and the sealing of the surfaces of epithelial sheets.

i. Gap junctions. Like other cell junctions, gap junctions provide physical linkage between the membrane of adjacent cells.

They are thought to be the basis of cell-cell communication (reviewed by Pitts et al., 1985). These structures are regarded as the site of ionic and thus electrical conductance and metabolic coupling between cells (Lee et al., 1987).

Revel and Karnovsky, (1967), demonstrated a region of close contact with a 2-4 nm space between oppositing membrane of liver or cardiac muscle cells. It was found that the gap junction particles in both crayfish septate axon (Peracchia, 1973) and mouse liver (Baker et al., 1983, Unwin and Zampighi, 1980) are composed of six subunits with a central pore. The major gap junction protein which represents each of the six subunits is a protein of 25-30 Kd molecular weight (Robertson, 1981, Gros et al., 1983). However, there are reports suggesting that amino acid sequences of the 25-35 Kd gap junctions proteins from varying tissues may be quite different (Nicholson et al., 1983; Gros et al., 1983).
Direct evidence for a developmental role for gap junctional communication during embryogenesis has been provided by the intracellular injection of antibodies raised against the major gap junction protein from isolated rat liver gap junctions (Lee et al., 1987). In the amphibian embryo, these antibodies block both dye transfer and electrical coupling between cells, and embryos reared with a clone of communication incompetent cells, develop pronounced patterning defects in structures derived from the antibody containing region (Warner et al., 1984).

ii. Desmosomes (zonula adherence). Are intercellular junctions, considered to play an important role in epithelial cell adhesion (Staehelin, 1974).

Ultrastructurally, desmosomes possess electron-dense subplasmalemmal membrane plaques to which intermediate filaments appear to attach (Kelly, 1966). The desmosome is structurally flat and roughly disk-shaped with an intercellular space of 25 to 35 nm (Kelly, 1966). The intercellular space separating the two opposed membrane of the desmosome is considered to be filled with adhesive

material and often contains an electron-dense midline (Staehelin, 1974).

A number of cytoplasmic components of desmosomes have been characterized. These include the desmoplakins which are polypeptides located in the region of the desmosomal plaque with which intermediate filaments appear to associate (Jones and Goldman, 1985).

In bovine muzzle epidermis the major glycosylated polypeptides of desmosome appear to be between 160 and 165 Kd (desmoglein 1 and 3) and polypeptides of approximately 115 and 130 Kd (desmoglein 2, band 4a and 4b and desmocollins) (Mueller and Franke, 1983, Cowin et al., 1984). Fab fragments of antibodies directed to these later proteins inhibit desmosome formation in cultured cells (Cowin et al., 1984).

iii. Tight junctions (zonula occludence). These were described as the most luminal element of the junctional complexes of various epithelia, characterized by fusion of adjacent cell membrane (Farquhar and Palade, 1963). In most epithelia the tight junction forms a continuous belt around the cell that is usually impermeable to electron-opaque tracers (Goodenough and Revel, 1970, Friend and Gilula, 1972).

It has not been determined whether tight junctional strands visible in freeze-fracture are composed of pure proteins, pure lipids, or lipids stabilized by membrane proteins. The treatment of cells with protein synthesis inhibitors produced conflicting results concerning the formation of tight junctions. However, Griepp et al., (1983) presented a convincing evidence that plasma membrane proteins are required for the formation of tight junctions.

On the other hand, it has been suggested that intramembranous inverted lipid micelles may represent the basic structural elements of tight junctions (Kachar and Reese, 1982).

Van Meer et al., (1986), showed that the tight junction does not

allow lipid molecules to diffuse from one epithelia to the next, suggesting that there is no fusion between the opposed plasma membranes, and that the tight junction is not formed by a hexagonal cylinder of lipids.

1.3.2-2. Cell-substratum adhesion.

The cell substratum interaction is one of the most widely investigated phenomena of cell biology for the following reasons:

a. The majority of cells derived from solid tissues are unable to grow suspended in a fluid medium (unless they are transformed). They must be provided with a solid surface to which they can adhere and spread, otherwise they stop growing.

b. In medical applications, especially in relation to prosthesis, it is necessary to establish non-adhesive surfaces for blood compatibility, or adhesive surfaces for cases where interactions with tissues are required (eg. bone implantation).

c. The cell-substratum contact is also important in the understanding of the mechanisms of cell interaction.

The identification of molecules involved in cell-substratum adhesion was based on four main approaches (see Yamada, 1983 for review).

i. Immunological. In this approach, anti-cell surface antibodies that inhibit adhesion are produced and characterized.

ii. Analysis of soluble adhesive factors.

iii. Examination of substrate-attached materials after detaching cells with chelating agents or other methods.

IV. Another approach to analyze cell interactions with substrate is to examine for novel biological activities of molecules released by cells into culture medium.

1. Extracellular matrix molecules. A wide range of glycoproteins have been described as mediating cell-substratum adhesion. Among these, is fibronectin (Yamada and Olden 1978) which was the first molecule to be described as being required for fibroblastic cell

adhesion.

Since then, a range of other molecules have been described, which include, collagen (Kleinman et al., 1981 for review) and laminin (Timpl et al., 1979; Martin and Timpl 1987 for review).

2. Cell surface molecules. The ligand-receptor model of cell-substratum adhesion is based upon the idea that specific cell surface receptors interact with specific ligands on the substratum, and that substrata coated with proteins or other materials for which cells have no receptors are not able to support adhesion (Grinnell, 1978).

In the last few years a number of matrix adhesion receptors of the plasma membrane have been described. These are the integrin or cytoadhesin family which are comprised of a number of heterodimeric receptors of ~140 Kd in molecular mass (Hynes, 1987).

The integrin superfamily are thought to include receptors for fibronectin, vitronectin (Pytela et al., 1985 a & b), collagen and laminin (Takada et al., 1988, Wayner and Carter 1987).

However, it is unlikely that the above evidences are the sole explanation of the mechanisms of adhesion, it is clear that there are other structures that may play an important role in cell adhesion. These include proteoglycan (Culp et al., 1986), carbohydrate residues of glycoproteins and glycolipids, gangliosides (Kleinman et al., 1979; Yogeeswaran, 1981), tenascin or hexabrachion (reviewed by Erickson and Bourdon 1989) and the platelet glycoprotein thrombospondin (Lahav et al., 1984; Frazier, 1987).

3. Thrombospondin (TS).

It was discovered in 1971 in human platelet membranes (Baenziger et al., 1971) using both, polyclonal and monoclonal antibodies. It has been confirmed that it has a role in platelet aggregation (Dixit et al., 1985, Gartner et al., 1984).

Thrombospondin is a multidomain or modular glycoprotein which has discrete regions of relatively stable protein structure that

contain binding sites for several proteins found in the extracellular matrix such as fibronectin, collagen, heparin and laminin, and some found in the plasma such as fibrinogen, plasminogen and histidine rich glycoproteins (Frazier, 1987 for review).

The native molecular mass of trimeric TS is ~450 Kd with a subunit mass of about 150 to 185 Kd based on sodium dodecyl sulphate gel electrophoresis under reducing condition (Lawler et al., 1978).

Thrombospondin was found using immunofluorescence localization techniques, in a variety of extracellular locations in human tissues including blood vessels, muscle, skin, kidney and glandular epithelium (Wight et al., 1985). Jaffe et al., (1983), demonstrated that cultured fibroblasts synthesize and secrete thrombospondin and incorporate it into the extracellular matrix.

This widespread expression of thrombospondin and its appearance as a component of the extracellular matrix have led to the suggestion that it might have a role analogous to that of fibronectin and laminin in cell attachment, mobility and perhaps differentiation (Frazier, 1987).

1.4. Cell activation and adhesion.

Beside the above evidence (physico-chemical or molecular) in explaining mechanisms of cell adhesion, it has been known that the adhesiveness of leukocytes and platelets can be changed from nonadhesive to a very strong adhesive state to each other and to many surfaces in a very short time (see later for details). The biologically active factors that produce such changes, include arachidonic acid metabolites (PGs, LTs, TXs), substances which stimulate their production, as well as agents affecting diacylglycerol (DAG) and inositol trisphosphate (IP_3) pathways (see Curtis, 1987 for commentary).

In addition to myeloid cells, evidence has emerged showing that

the sponge *Microciona prolifera* cells can have their adhesion activated using agents which mobilize calcium and activate protein kinase C (Weissmann et al., 1986). Recently it was demonstrated that there is a correlation between an increase in protein kinase C activity and formation of cell-cell contact in human carcinoma cell line (Nagao et el., (1989). Chinese hamster ovary cells treated with phorbol ester were shown to produce a remarkable increase in their ability to adhere to fibronectin (Danilov and Juliano 1989). This may suggest that activation phenomena is of general significance in cell adhesion.

Accordingly, it is of great importance to review the activation events in blood cells and in particular the pathways regulating their adhesion.

1.4.1 Leukocyte activation and the process of activation.

1.4.1-1. General.

External signals detected by surface receptors are translated into a limited repertoire of intracellular second messengers. The biochemical basis of the transduction of extracellular signals into intracellular events such as the release of second messengers which in their turn trigger a variety of cellular responses (Nishizuka, 1984 & Berridge and Irvine 1984) is a subject of great interest.

Inositol phospholipids have attracted great attention from researchers studying the activation of cellular functions and proliferation. The response of inositol phospholipids to the activation of the cell surface receptor was first recognized by Hokin and Hokin (1953), who showed that acetyl choline induces a rapid incorporation of ^{32}P into phosphatidyl inositol and phosphatidic acid in pancreatic slices. In the action of group of hormones, neurotransmitters or biologically active substances, a signal induced degradation of phospholipids may generate important intracellular second messengers that function differently from cAMP

(Kikkawa and Nishizuka 1986). The two first second messengers released as a response to extracellular signals are now known to be IP_3 and DAG which constitute a bifurcating signal that is attracting enourmous interest because of their involvement in the control mechanism of many cells (Berridge, 1987) (see Fig.1.1).

The precursor molecule used by the receptor mechanism to release IP_3 and DAG is believed to be phosphatidyl inositol 4,5 bis phosphate (PIP_2) releasing IP_3 to the cytosol and leaving DAG within the plane of the membrane (Fisher, et al., 1984) under the action of phospholipase C.

The primary function of IP_3 is to mobilize calcium from intracellular stores to constitute an IP_3 /Ca^{+2} pathway, whereas DAG stimulates protein kinase activity to form DAG /PKC pathway (Berridge, 1984 & Berridge, 1986).

IP_3, beside its role in stimulating the release of calcium during cell activation, might play an equal role in regulating the resting or basal level of calcium (Prentki et al., 1985).

The first direct evidence that IP_3 functions to mobilize intracellular calcium was obtained (Streb et al., 1983) using permeablised pancreatic cells. Similar results have now been reported for many other permeablised or intact cells. IP_3 binds to a membrane-bound intracellular receptor which is believed to reside in the smooth endoplasmic reticulum (Spat et al., 1986). The occupancy of this receptor releases calcium into the cytosol via an as yet uncharacterised mechanism, which may however involve a guanine nucleotide regulatory protein (Dawson, 1985). Recently Chadwick et al., (1990), isolated and characterized an IP_3 receptor from smooth muscles. The purified receptor is of 224 M_r, and the negative-staining electron microscopy reveals that the receptor is a large pinwheel-like structure having surface dimensions of 250 x 250 Å.

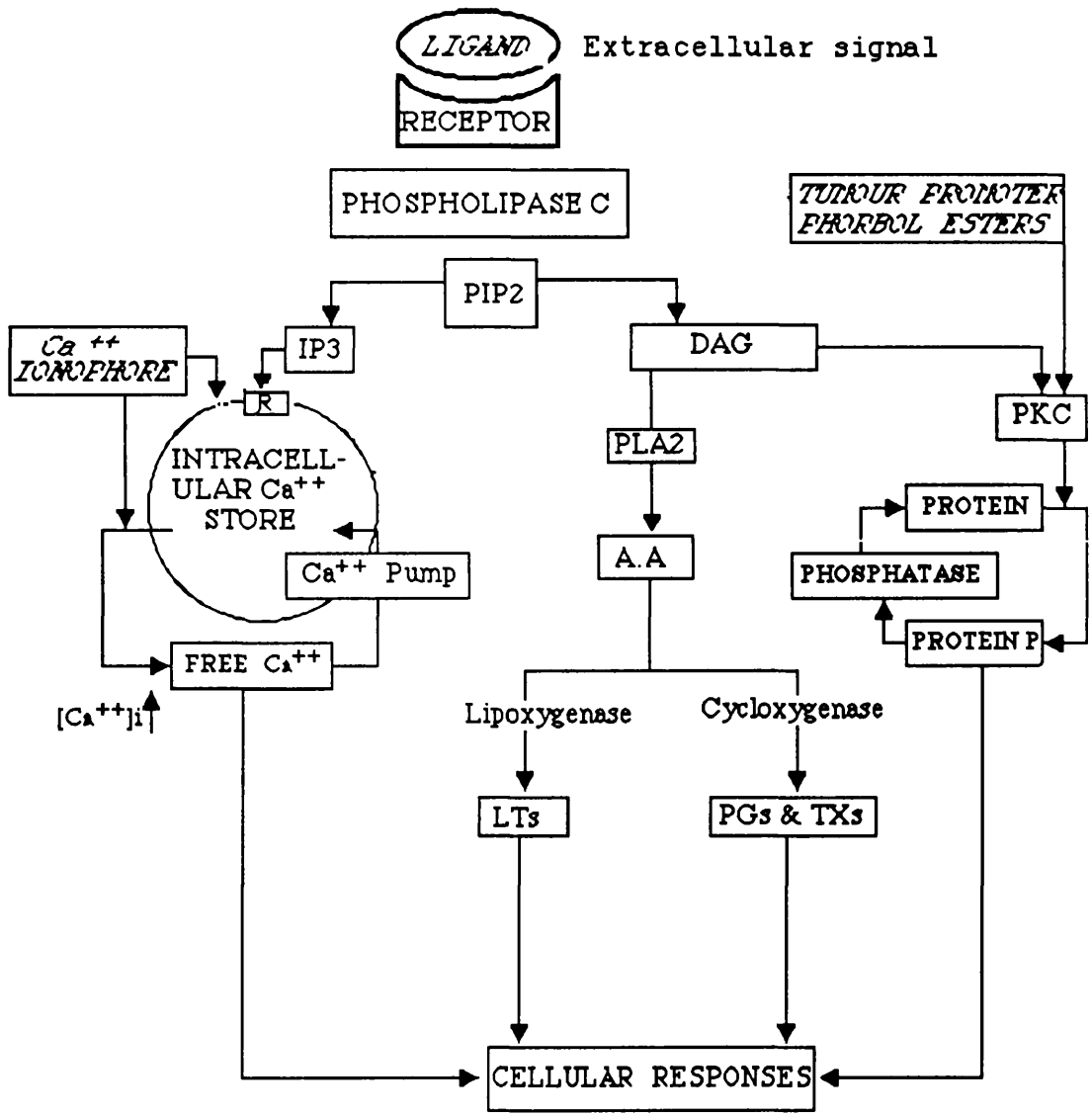


Fig.1.1. Inositol phospholipid turnover and signal transduction. From Nishizuka, (1984) with modifications.

Abreviations: PIP_2: Phosphatidyl inositol bisphosphate; DAG: Diacyglycerol; IP_3: Inositol trisphosphate; PKC: Protein kinase C; PLA_2: Phospholipase A_2; A.A: Arachidonic acid; PGs: Prostaglandins; TXs: Thromboxanes; LTs: Leukotriene; R: Receptor.

The neutral molecule of DAG that remains within the plane of the plasma membrane activates PKC (Nishizuka, 1986). It was suggested that the rise in Ca^{2+} may contribute to the activation of PKC (Dougherty and Niedel 1986) perhaps by increasing the binding affinity of the enzyme for diacylglycerol.

1.4.1-2. Lymphocyte activation.

Microbial invasion, injury, immunological reactions, neoplastic changes and inflammatory processes, continually challenge the host´s ability to survive. The host faced with either exogenous or endogenous insults, responds with a series of dramatic changes which are characterized by alteration in metabolic, endocrinologic neurological and immunological functions (Dinarello et al., 1986).

The two major lymphocyte populations in the immune system are the B- and T-lymphocytes. This two types serve different immunological functions. Antigens can induce a specific immune response which results in the release of antibodies by B-lymphocytes and development of cytotoxic, suppressor and helper T-cells.

It has been proposed that the activation of T-lymphocytes by antigen is mediated by an early rise in cytosolic calcium concentration (Tsien et al., 1982; Hesketh et al., 1983). However, it has not been possible to mimic antigen or mitogen induced mouse lymphocyte activation with calcium ionophores that bypass receptor mediated process (Hesketh et al., 1983; Hesketh et al., 1977).

From other evidences, in other systems, it has been established that the rise in intracellular calcium is preceded by the breakdown of PIP_2 to two second messengers IP_3 and DAG as it was mentioned earlier. Therefore ligand induced cellular response follows a rise in cytoplasmic calcium concentration and protein kinase activity. Truneh, et al., (1985) confirmed that the calcium ionophores A23187 and ionomycin do not activate mouse T-lymphocytes. However, either

one in combination with the phorbol ester (TPA) which is structurally related to DAG, induces in lymphoid cell populations the expression of receptors for interleukin-2 (IL-2), the secretion of IL-2 and cell proliferation as measured by ^{3}H-thymidine uptake, features also typical of antigen activation.

An analysis of the requirement for calcium in the lysis of target cells by cloned cytotoxic T-lymphocytes(CTL) was carried out by Ostergaard and Clark, (1987). It was observed that an influx of calcium into the CTL concomitant with target cell binding in direct antigen-specific lysis. However, these influx is not observed in CTL during lectin-mediated lysis or nonspecific lysis by PMA induced CTL. This may suggest (Ostergaard and Clark, 1987) that calcium is not always required for the activation of the lytic pathway in CTL, although calcium may be absolutely required for other CTL functions such as IL-2 production or expression of the IL-2 receptors.

One of the positive biochemical consequences of PKC activation is the increase of metabolic and proliferative activity of stimulated lymphocytes which is believed to depend largely on an increase in transport of various metabolites including glucose, an increase in ornithine decarboxylase activity as the key enzyme for polyamine synthesis and control of cell proliferation and an increase in glycolytic activity as a source of energy (see Droge, 1986 for review).

Wilkinson et al., (1988) reported a change in shape and locomotion of human blood lymphocytes after exposure to phorbol esters. The change in shape was independent of extracellular calcium as is PKC activity. They reported also that the shape was not detectably inhibited by PKC inhibitors. They suggest that phorbol ester induce immediate change in the shape followed by an efficient locomotion after prolonged exposure, possibly because phorbol esters, like

other growth activators stimulate events during G phase of growth such as gene expression and synthesis of new proteins necessary for locomotion.

Results of several investigators suggest that products of arachidonic acid metabolism have modulatory effects on cellular immunity. It was reported that a newly isolated compound derived from the oxygenation of arachidonic acid (lipoxin A.) inhibits the cytotoxic activity of human natural killer cells (Ramstedt et al., 1985). Conti et al., (1986) also found that indomethacin, the cyclooxygenase inhibitor, is able to enhance in a dose-dependent way, natural killer cell activity *in vitro*, and the effect is potentiated in combined pretreatment with indomethacin and human recombinant interferon α or γ. This suggests that the inhibition of PGs synthesis increases natural killing (Bray et al., 1983). Later, Conti et al., (1987) confirm their previous finding, in that pretreatment of peripheral blood mononuclear cells with this drug also augments the stimulating effect of human recombinant interferon α (hr-IFnα) or γ on cytolysis mediated by these cells even when the effector cells were simultaneously pretreated with antiserum against IL-1. This finding demonstrates prostaglandin action in modulating lymphokine-dependent response. It is in fact well documented that the ability of PGE_2 to modify T-cell function and in particular to activate suppressor mechanisms (Dinarello et al,. 1983).

1.4.1-3. Polymorphonuclear leukocyte activation.

1. General activation.

A common histological feature of acute inflammation is the accumulation of PMN leukocytes at the site of the reaction. The stimulation of these cells in inflamed tissues is followed by the release of several inflammatory substances such as lysosomal enzymes (Baggiolini et al., 1979), arachidonic acid metabolites

(Higgs et al., 1979) and toxic oxygen derivatives (Fantone & Ward 1982).

PMN also can be stimulated to generate superoxide anions (O_2^-), aggregate and degranulate by a variety of ligands, such as the chemotactic peptide fMLP, immune complexes, the complement component C5a, and the lectin concanavalin A (Korchak et al., 1984). These ligands trigger an increase in cytosolic calcium as part of the activation sequence (White et al., 1983; Shell-Frederick, 1984).

The increase in intracellular calcium is a consequence of the breakdown of PIP_2 to DAG and IP_3 in which DAG activates PKC and IP_3 mobilizes cell-associated calcium. The action of IP_3 and calcium sequestering pools have been characterized in digitonin-permeabilised human neutrophils using a calcium-selective electrode (Prentki et al., 1984). Both mitochondrial and nonmitochondrial pools act in concert to restore the ambient calcium concentration following its elevation, but IP_3 released calcium accumulated in the ATP-dependent pools and had no effect on the mitochondria. Under experimental condition in which the ATP-dependent calcium influx was blocked, the addition of IP_3 resulted in a very large calcium release from nonmitochondrial pools (Prentki et al., 1984).

Protein kinase C and calcium may act synergistically to trigger biological responses such as the release of oxygen radicals in neutrophils.

In order to demonstrate the necessity of calcium for neutrophil activation, Korchak and his coworkers (1984 1988a) have chosen three different stimuli that trigger O_2^- production but elicit different patterns of calcium mobilization. They found that fMLP triggers a rapid increase in cytosolic calcium of intra and extracellular origin. In contrast, the lectin Con A triggers a slow but substantial increase in cytosolic calcium which comes

principally from the extracellular milieu as judged by ^{45}Ca uptake, while the tumour promoter PMA does not elicit any increase in cytosolic calcium. The peptide fMLP and Con A triggered elevation of DAG but PMA a direct activator of PKC, bypassing the normal signalling pathway and triggering O_2^- generation without an increase in calcium, DAG, IP_3 or PIP_2 breakdown.

These results suggest that the breakdown of PIP_2 and generation of IP_3 are associated with mobilization of intracellular calcium which is not otherwise an absolute requirement for the end response of oxygen peroxide generation.

A role for protein PKC has been proposed in the activation events such as O_2^- production, since activators of PKC, phorbol esters and DAG analogues have been shown to activate O_2^- generation (Cox et al., 1986). Enhanced phosphorylation of a number of proteins has been demonstrated in neutrophils activated by diverse stimuli (Hayakawa et al., 1986) and PKC activity has been demonstrated in cytosol and particulate fractions of neutrophils (Melloni et al., 1985).

In assessing the role of PKC in cellular response, it is essential to establish that the removal of the signals for activating PKC (DAG and Ca^{2+}) prevents the end responses such as degranulation, proliferation and oxygen radical release.

Korchak et al., (1988 b), studied the correlation of calcium, DAG and phosphatidic acid production with O_2^- generation using fMLP, Con A, LTB_4 and ionomycin. They demonstrated that the elevation of cytosolic calcium was not a sufficient signal for O_2^- generation, but its requirement could not be dissociated from a requirement for DAG. They concluded that activation of O_2^- generation was associated with rapid elevation of cytosolic calcium and DAG. The removal of these signals inhibited the response. Oxygen generation was also associated only with ligands that trigger phosphatidic acid, DAG

and calcium release. Therefore, the correlation of O_2^- generation with phosphatidic acid production, might reflect a requirement for a specific pool of DAG that is a potent activator of PKC and that is capable of being metabolized to PA, either because it is the correct molecule species of DAG or because it is generated in the appropriate location, presumably the plasmalemma (Korchak et al., 1988 b).

Locomotion is also a part of the activation events, in that low concentration of chemotactic factors such as fMLP, LTB_4, complement-derived C5a are required to induce optimal migration of PMN, but higher concentrations are needed to stimulate their optimal respiratory burst or degranulation (Snyderman and Pike, 1984).

Locomotion of PMN as well as other cells depends on their attachment to the surface they are moving on, since excessive adhesion to the substratum immobilizes the cell and diminished contact results in decreased movement.

Not only leukocytes move, but they regulate their movement in order to reach the inflammatory site.

Three morphological states of PMN migration have been described (Malech et al., 1977). These are, random migration, activated random migration and directed migration (chemotaxis). In order to ascertain whether or not PKC is involved in spontaneous and /or directed migration of PMN, the effect of the newly described inhibitor of PKC (H-7) on this migration was tested (Gaudry et al., 1988). Preincubation of PMN with H-7 (50-400 μM) inhibited spontaneous, directed and the speed of the migration. This indicates that H-7 inhibited both the spontaneous and stimulated migration induced by fMLP, LTB_4 and by activated serum, by slowing down this migration. All the effect of H-7 was reversed by washing PMN after treatment. These finding indicate that PKC, inhibitable by H-7 is involved in a mechanism controlling the speed of PMN

protein kinase C inhibitor H-7 is by itself a potent activator of several neutrophil functions that include shape changes, actin polymerization, pinocytosis, increased adhesion and chemokinesis (Keller et al., 1990).

As mentioned earlier in this section, the activation of PMN is accompanied by calcium mobilization and membrane phospholipids remodeling. There is an increasing evidence for a parallel arachidonic acid turnover as a result of stimulation. The chemotactic fMLP, calcium ionophore (Rubin et al., 1981) and phorbol dibutyrate (Kramer et al., 1984) have been reported to stimulate a parallel arachidonic acid turnover and enzyme secretion by a calcium-dependent process. Phorbol esters, beside their ability to activate PKC (Gennaro et al 1986), have been reported to stimulate PLA_2 and arachidonic metabolism in several cell types (Beaudry et al., 1982) including neutrophils (Hirata et al., 1979).

It was shown that PMN leukocytes release products of arachidonic acid metabolism as a consequence of phagocytosis (Goldstein et al., 1978). LTB_4, an arachidonic acid metabolite, have been shown to be synthesized by eosinophils after induction with either calcium ionophore (Weller et al., 1983), opsonized zymosan (Bruynzeel et al., 1985) or IgG-coated sepharose particles (Shaw et al., 1985). A role for PGE_2 and LTB_4 have been also proposed in the activation of neutrophils (Lew et al., 1984).

It is therefore clear, that calcium/protein kinase C pathway, prostaglandins and leukotrienes represent mediators or modulators that are physiologically involved in the regulation of many cellular functions including cell adhesion (see next section).

2. Activation of PMN leukocyte adhesion.

The adherence of circulating leukocytes to the walls of small blood

vessels is an early event in nearly all forms of inflammation (Grant, 1973). Under normal conditions, blood-born PMN flow freely through the vessels or at most adhere briefly to the endothelial surface (Atherton and Born 1972).

During acute inflammation, the PMN marginate along the vessel wall and subsequently emigrate along the vessel through the inter-endothelial junctions to accumulate at the site of the injury or inflammation (Hoover et al., 1978).

Factors responsible for provoking adherence of PMN to endothelial surfaces are largely unknown. It has been suggested however, that the same factors that stimulate directed migration of PMN towards site of inflammation also enhance their attachment to endothelial cells (Hoover et al., 1978).

Several investigators have reported that the complement C5-derived peptides, as well as other chemotactic factors, increase adherence of human PMN to cultured endothelial cells (Hoover et al., 1978, 1980; Hoover, 1978; Gimbrone et al., 1984; Charo et al., 1985).

Hoover and his colleague for example, have reported that adherence of human PMN to bovine aortic endothelial cells was enhanced by trypsinised human C5, zymosan-activated human serum, or fMLP (Hoover et al., 1980). Further evidence has been presented suggesting that the effect of chemotactic factors on PMN adhesion depends on their concentration (Keller et al., 1981).

Charo et al., (1986), used a new sensitive centrifugation assay to examine the effects of the chemotactic peptides on both the extent and strength of PMN adherence to monolayers of human umbilical vein endothelial cells. They found that low chemotactic concentrations significantly reduced PMN adherence to endothelial cells, while, higher concentrations of both peptides C5a and fMLP enhanced PMN adhesiveness in a concentration-dependent manner. The precise mechanism by which high concentrations of C5a and fMLP enhanced PMN adhesiveness is unclear. However, surface charge

(Gallin, 1980), PMN granule constituents (Oseas et al., 1981; Bockenstedt and Coetzl, 1980), oxygenation products of arachidonic acid (Spagnuolo et al., 1980) and plasma membrane proteins (Todd et al., 1984; Arnaout et al., 1985) might be important.

Evidence in favour of the involvement of surface charge was obtained by Hoover, (1978). He pointed out that treatment of either the endothelial cells or PMN with neuraminidase resulted in an increase in adhesion. Additional evidence is provided by his data from calcium-magnesium-free experiment which resulted in reduced adhesion.

Evidence has been presented concerning membrane proteins involvement in that chemotactic factors may augment PMN adherence by provoking the translocation of adhesive glycoproteins from an intracellular pool to the cell surface (Todd et al., 1984; Berger et al., 1984).

Arachidonic acid metabolites have been reported by many investigators to have a modulatory effect on the adhesive interaction between PMN leukocytes and endothelial cells.

It was demonstrated that lipopolysaccharide (LPS) stimulates the generation of a low molecular weight, labile and dialysable substance capable of enhancing the adherence of unstimulated PMN (Spagnuolo et al., 1980). Inhibitors of both cyclooxygenase and thromboxane synthase reduced the increase in PMN adherence induced by arachidonic acid. This suggests an involvement of an arachidonic acid product other than prostaglandin E_1, E_2 or $F_{2\alpha}$, because they did not increase PMN adherence and were not detected in significant quantities in supernatant of PMN exposed to LPS (Spagnuolo et al., 1980). A 10 fold increase in Thromboxane A_2 (TXA_2) was detected after exposure of PMN to LPS. Using specific rabbit antibodies against TXA_2, LPS enhancement of PMN adherence was inhibited which suggests strongly a primary role for TXA_2 as a mediator of the observed increase in adherence. They concluded from their

experiments that LPS stimulates PMN to produce TXA_2 which enhances their adhesiveness.

Hoover et al., (1984), reported that leukotriene B_4 (LTB_4) augmented neutrophil/ endothelial adhesion. The LTB_4 dependent adherence of PMN to endothelium is selectively mediated by endothelial cells, since preincubation of PMN with LTB_4 did not either increase PMN attachment to plastic substratum or to untreated endothelial cells.

Later, McIntyre et al., (1986), found that LTC_4 and D_4, arachidonic acid metabolites, stimulate human endothelial cells to synthesize platelet activating factor and bind neutrophils. LTC_4 and D_4 induced also the adherence of human neutrophils to the endothelial cell monolayer. They indicated that the adhesion was an endothelial cell-mediated process, because neither LTC_4 nor D_4 stimulated the adhesion of PMN in the absence of endothelial cells.

The adherence of PMN to nylon has been demonstrated (MacGregor et al., 1978) to be an *in vitro* correlate of adherence to endothelial cells. McGillen et al., (1980), have investigated the *in vitro* effect of PGI_2, hydrocortisone and zymosan on PMN adherence to nylon. Both PGI_2 (ng/ml) and hydrocortisone (5mg/ml) inhibited base line adherence of PMN, in contrast to zymosan-treated plasma which augmented retention of PMN by nylon columns. The zymosan mediated effect, was blocked by incubating plasma with anti-serum to C5a or incubation with PGI_2 or hydrocortisone. PGE_1, D_2 and $F_{2\alpha}$ did not alter the base line adherence, though in a previous study, PGE_1, E_2 and $F_{2\alpha}$ have been shown to inhibit PMN aggregation induced by the synthetic chemotactic peptide fMLP (O'Flaherty et al., 1979a).

The mechanism by which leukocytes adhere to artificial surfaces is not fully understood. However, there is a suggestion of the involvement of plasma components adsorbed to the surfaces.

An experiment was set up to test the influence of several purified plasma proteins on the attachment of PMN to cuprophane

surface (Chuang et al., 1980). Their study demonstrates that albumin, fibrinogen, and haemoglobin inhibit PMN adhesion to cuprophane (cellulose), while immunoglobulin G (IgG), IgM and thrombin enhance this reaction. They suggest that the inhibitory effect of fibrinogen and the enhancement of IgG on PMN adhesion to cuprophane are likely due to modification of the cuprophane surface by adsorbed proteins. They found also that prostacyclin (PGI_2) inhibits PMN adhesion (induced by IgG) to cuprophane. Other prostaglandins (E_1, E_2, B_2, $F_{1\alpha}$) at concentrations up to 100 μM did not exert significant inhibition of the same reaction.

The phorbol ester PMA, has been reported to stimulate increased human neutrophil adherence to plastic surfaces and confluent cultured endothelial cells (Webster et al., 1986). The concentration of PMA required to affect increased adherence was similar to that required to cause maximal release of the specific granule-containing enzyme lysozyme and maximal generation of superoxide anion. It was observed that neutrophils require exposure to PMA for at least 5 mins before irreversible adherence-promoting activity occurs (Webster et al., 1986). They suggest that certain metabolic events are required for increased adhesiveness.

In contrast to the ability of cytochalasin B to potentate neutrophil aggregation (O'Flaherty et al., 1979b), Webster et al., (1986) showed that pretreatment of neutrophils with cytochalasin B did not modulate (increase or decrease) PMA-induced increase in adherence. PMA-stimulated neutrophils, spread widely on the endothelial monolayer or on plastic, whereas, neutrophils treated with cytochalasin B before PMA, rounded up and failed to spread (Webster et al., (1986). Despite the obvious difference in morphologic features, there was no difference in the adherence of the neutrophils. This suggests that the amount of surface contact between neutrophils and substrate alone is not a major consideration in adhesion, other factors such ionic forces,

secreted granular constituent and cell surface proteins must constitute the major determination of adherence (Webster et al., 1986). The failure of cytochalasin B to influence PMA stimulated neutrophil adherence, indicate also that intact cytoskeletal microfilaments are required neither for initiation of neutrophil adherence nor for maintenance of neutrophil adherent.

The PMN leukocytes have the capacity to release a variety of substances including oxygen radicals which might cause tissue damage.

In this respect Chopra and Webster (1988), evaluated the ability of PGE_1 (1-100μM) to protect endothelial cells from neutrophil injury induced by C5a or PMA. The protective effect was more effective when treating neutrophils rather than endothelial cells. They demonstrated also that there was a concentration dependent inhibition of neutrophil adherence to plastic and endothelia following stimulation with PMA, human C5a or fMLP. Using fluorescence flow cytometry they measured the expression of heterodimer surface glycoprotein MO1 (Mac-1) previously shown to be associated with increased granulocytes adherence (Todd et al., 1984, Harlan et al., 1985). There was no significant inhibition of PMA or fMLP stimulated Mo1 expression in PGE_1-treated neutrophils compared with the neutrophils stimulated in the absence of PGE_1. This finding indicates that PGE_1 has the ability to inhibit neutrophil-mediated endothelial cell injury by modulating adherence by mechanisms independent of Mo1 antigen expression on neutrophil surface. Previous studies have shown a correlation between PGE_1 modulation of neutrophil functional responses and increased cAMP levels (Weissman et al., 1976; Marone et al., 1980). Bryant and Sutcliffe (1974) also found that increased intracellular cAMP levels suppressed unstimulated granulocyte adherence to glass capillary tubes. These findings suggest that PGE_1 may cause inhibition of stimulated neutrophil adherence as a result of increased intracellular levels of cAMP. In contrast, a significant

decrease in the expression of Mo1 antigen on the surface of unstimulated but PGE_1 treated neutrophils was found (Chopra and Webster 1988). This indicates that the mechanism of inhibition of basal adherence of neutrophils to plastic or endothelial cells by PGE_1 is related to the expression of Mo1 antigen.

1.4.2. Platelet activation.

Adherence of blood platelets to the vessel wall is an early event in the formation of intravascular thrombi and perhaps in the development of atherosclerosis (Ross & Majerus, 1986).

Under normal circumstances platelets circulate in a quiescent state but, when activated, platelets can adhere to many surfaces. Those that they usually come in contact with are the lining of the blood vessels and the surface of other blood cells. However, many experiments have been performed on the interaction of platelets with artificial surfaces, because such adhesion is important in vascular prosthesis (Gordon, 1980). The success of such prosthesis is however, dependent on the degree to which platelets adhere to them (Gordon, 1980).

The stimulation of platelets to secrete and aggregate by agonists such as thrombin and platelet activating factor, is associated with a rapid increase of the cytoplasmic free calcium concentration (Hallam et al., 1984; Rink et al., 1982), and the hydrolysis of the phosphodiester bond of phosphatidyl inositol 4,5-bis-phosphate (Vickers et al., 1984). The products of this reaction, DAG and IP_3 appear to play important roles in various cellular activation processes.

Diacylglycerol acts as a second messenger to stimulate calcium /phosphatidylserine-dependent protein kinase C by increasing the affinity of the enzyme-lipid complex for calcium, so that the enzyme can be activated even at resting levels of calcium concentration (Kishimoto et al., 1980). Diacylglycerol is further

metabolized to phosphatidic acid by diglycerol kinase (Lapetina et al., 1981) or hydrolysed to release arachidonic acid, stearic acid and glycerol by diglyceride lipase (Bell and Majerus, 1980).

IP_3, has been implicated as a second messenger that causes the release of calcium from intracellular storage sites in various cells (Streb et al., 1983, Joseph et al., 1984), including platelets (O'Rourke et al., 1985).

Zavoico et al., (1985), reported that the phorbol ester, PMA inhibits calcium mobilization, PIP_2 breakdown, phosphatidic acid formation and secretion caused by thrombin stimulated human platelets. They suggested that the protein kinase pathway can exert feedback inhibition on receptor-mediated platelet activation subsequent to its initial role in promoting secretion.

Activation of PLA_2 on the other hand, results in the direct release of arachidonic acid primarily from phosphatidylcholine (Ballou and Cheung, 1983).

Arachidonic acid released from either pathways, serves as the precursor molecule for the synthesis of PGs, TXs and LTs.

There is a considerable interest in the effect of PGs on blood platelets, partly because the synthesis of PGs by stimulated platelets is important in heamostasis, and partly because platelets can be used as models to investigate the action of PGs at a cellular level (Flower, 1975). They were found to exert different effects on platelets. Prostaglandin E_1 is a potent inhibitor of platelet aggregation (MacIntyre and Gordon, 1974) and PGD_2 is an even more effective inhibitor of human platelet aggregation. Prostaglandin E_2 was found to inhibit platelet aggregation at concentrations higher than 10^{-6} M (Bruno et al., 1974).

It was shown that ionized calcium antagonized the inhibitory effect of PGE_1 on ADP-induced aggregation of human platelets (Vigdahl et al., 1969). MacIntyre and Gordon, 1975 demonstrated

that this antagonism is not limited to PGE_1 or to platelet aggregation induced by ADP, since inhibition of collagen-induced aggregation of human and rat platelets by PGE_1, PGE_2 and PGD_2 was less in heparin plasma-rich platelets than in the citrated one. They showed also that PGE_2 inhibited pig platelet aggregation in citrated plasma-rich platelets (PRP), but induced it directly in heparin plasma-rich platelets.

Moreover, the endoperoxides,which can themselves induce platelet aggregation and secretion are converted mainly to TXA_2 which is highly labile, but is an extremely potent platelets stimulant and vaso-constrictor (Hamberg et al., 1975). Platelet TXA_2 has been implicated as an endogenous mediator of aggregation, in that, inhibition of its synthesis can reduce platelet aggregation *in vitro* (Whittle & Moncada 1983)

The vascular endothelium generates factors which modulate the homeostatic interaction between platelets and the vessel wall (Radomski et el., 1987). One of the most potent of these factors is prostacyclin (PGI_2), a product of the metabolism of arachidonic acid by cyclooxygenase (Moncada et al., 1976). Prostacyclin inhibits platelet aggregation and induces vasodilatation (Gorman et al., 1977).

It was demonstrated also that PGI_2 completely prevents the formation of thrombi and partially inhibits platelet spreading on the surface of the subendothelium (Higgs et al., 1978, Weiss and Turrito, 1979), and inhibited the adhesion of platelets to the subendothelium and collagen-coated surface of glass sticks (Cazenave et al., 1979).

Mazurov et al., (1988), investigated the effects of a stable prostacyclin analogue, carbacyclin on the interaction of platelets with collagen substrates differing in their ability to activate platelets. They demonstrated that carbacyclin in the concentration inducing 10-fold rise in platelet cAMP did not affect platelet adhesion to collagen of weak ability to activate platelets, but

reduced the adhesion to strong collagen. Carbacyclin inhibited also all morphological manifestations of platelet activation associated with adhesion, and inhibited massive spreading and aggregation on weak substrate stimulated by arachidonic acid and thrombin.

Endothelium-derived relaxing factor (EDRF) has been also reported to inhibit platelet aggregation (Furlong et el., 1987). Radomski et al., (1987) studied the pharmacological effects of nitric oxide and EDRF as inhibitors of platelet aggregation and compared their activity with that of prostacyclin.They demonstrated that EDRF and nitric oxide have identical pharmacological activity on platelets supporting the hypothesis that EDR factor might be nitric oxide.

The antiaggregatory effect of prostacyclin was shown to result from its ability to stimulate platelet adenylate cyclase and elevate intracellular cyclic-AMP levels (Gorman et al., 1977).

1.5. Aim of research.

One of the main interests of developmental and cell biologists over a long time ago, has been to achieve an understanding of the mechanisms by which cells are held together. Thus a great deal of interest has been devoted to develop new methods and treatments in order to understand these mechanisms.

In this respect many workers in this field have been looking to the role that plasma membrane molecules may play in cellular adhesion. Phospholipids in particular are amongst these molecules.

Previous work (Fischer et al., 1967; Curtis et al., 1975; Hoover et al., 1977) has shown that the lipid composition of the plasmalemma can be altered by taking advantage of the acyltransferase system to incorporate selected fatty acids into phospholipids of the plasmalemma.

Accordingly, I shall examine relation between plasma membrane phospholipid modification and cell adhesion using different fatty acids that vary in their hydrocarbon chain length and saturation.

In addition, I shall examine the activation phenomena in cell adhesion in cell types other than PMNs and platelets.

Work on white blood cells and platelets has shown that their adhesion is controlled by series of activation events, in particular that involving product of fatty acid metabolism and the protein kinase C pathway (Flower, 1975; Gordon, 1980; Nishizuka, 1984) (see earlier). In addition to myeloid cells, the adhesion of sponge *Microciona prolifera* cells were shown to be activated using agents which mobilize calcium and activate protein kinase C (Weissmann et al., 1986). Nagao et el., (1989), presented data showing a correlation between an increase in C-kinase activity and formation of cell-cell contact in human carcinoma cell line. It was demonstrated also that chinese hamster ovary cells treated with phorbol ester produced a remarkable increase in their ability to adhere to fibronectin (Danilov and Juliano 1989).

The question under investigation, is whether such phenomenon also occur in the control of adhesion and shape of tissue culture

such as baby hamster kidney fibroblasts.

Based on this hypothesis, the biologically active factors which include fatty acid metabolites as well as agents stimulating calcium/protein kinase C pathway were used and their effect on BHK cells was investigated.

Beside the above major theme, other minor themes were investigated. These include the following:

1. Does the method of preparation of cell suspension or the presence of serum in medium affects the results? It was shown by Curtis and McMurray (1986), that even brief exposure of cells to serum resulted in an adhesion dependent on serum.

2. Are the effects on spreading different from that on adhesion?

3. Are cells viable during the test?

Chapter II

Materials and Methods

II. Materials and methods.

2.1. Materials.

2.1.1. Cell line: Baby Hamster Kidney cells (BHK21 C13) and a mouse pulmonary capillary endothelial cell line (B10.D2) (isolated in the Department, 1984) were supplied frozen from departmental stock and recultured continuously (see Methods) in bicarbonate-buffered Eagles medium in 25 or 75 cm^2 polystyrene tissue culture flasks obtained from Falcon (Becton Dickinson UK. Ltd. Between Towns Road, Cowley, Oxford OX 43 LY) or Bibby (Stone, Staffordshire ST15 OSA).

2.1.2. Tissue culture dishes. 60x15 mm polystyrene tissue culture dishes obtained from Falcon (Becton Dickinson U.K. Ltd Between Towns Road, Cowley, Oxford OX 43 LY) and Nunclon (PB. 280, Roskilde, Denmark).

2.1.3. Media recipes.

1. Hepes saline(H.S).

Quantities for 5 litres .

NaCl	40 g
KCl	2 g
D-glucose	5 g
Phenol red (0.5 % solution)	10 ml
Hepes	11.90 g

pH adjusted to 7.2 with 5M NaOH.

Hepes is N-2 hydroxyethyl piperazine-N-2-ethane sulphonic acid (Boehringer Mannheim House, Bell lane, Lewes, East Sussex BN7 1LG).

2. Hanks Hepes(HH)medium.

Quantities for 5 litres

NaCl	4 0 g
KCl	2 g
D-glucose	5 g
Phenol red(0.5% solution)	10 ml

Hepes	11.92 g
$CaCl_2.2H_2O$	0.93 g
$MgCl_2.2H_2O$	1 g

pH adjusted to 7.5 with 5M NaOH.

Both Hepes saline and Hanks Hepes were dispensed in 100 ml aliquots, sterilized by autoclaving at 121°C for 20 mins and stored at 4°C

3. Bicarbonate Buffered Eagles Medium(ECT).

Total volume: 201 ml.

Sterile distilled water	134 ml
x10 concentrate Glasgow-modified Eagles medium (GMEM) (Gibco Ltd, Paisley Scotland)	16 ml
*GPSA	5 ml
7.5% $NaHCO_3$	6 ml
Calf serum (Gibco Ltd, Paisley Scotland)	20 ml
*TPB	20 ml

*GPSA: Glutamine (114 mM); penicillin(1905 units/ ml); streptomycin (1905µg/ml); amphotericin B (11.9µg/ml) (Flow Laboratories, Woodcock Hill Harefield Road Rickmansworth, WD3 1PQ).

*TPB:Tryptose phosphate broth. 147.5g TPB. (Gibco Ltd, Paisley, Scotland) dissolved in 5 litres double deionised water and pH adjusted to 7.4.

4. Serum(3%)Ham's F-10 medium.

Total volume 211.5 ml

22mM sterile Hepes water (5.25g/l)	180 ml
x10 concentrate nutrient mixture Ham's F-10 Medium (Gibco Ltd, Paisley Scotland)	20 ml
GPSA.	5 ml
Foetal calf serum (Flow laboratories, Harefield Road, Rickmansworth, WD3 1PQ)	6 ml
ITS.	0.5 ml

*ITS:insulin/transferrin/selenite supplement (prepared in the department of cell biology) dissolved in 0.1M acetate buffer at pH 2.0.

5. Serum-free Ham's F-10 medium.

Total volume: 205 ml.

22 mM Hepes water	180 ml
x10 concentrate nutrient mixture Ham's F-10 (Gibco Ltd, Paisley Scotland)	20 ml
GPSA.	5 ml

6. Formal saline(fixative).

Quantities for 1 litre.

NaCl	8.5 g
Formalin (formaldehyde 40%)	100 ml
Distilled water	900 ml

7. Kenacid blue(Coomassie blue).

Quantities for 1 litre

Kenacid blue (Sigma, Chemical Company Ltd, Poole, England)	1 g
Distilled water	430 ml
Methanol	500 ml
Acetic acid	70 ml

8. Silicone fluid(for coating glass to reduce adhesion)·

1% silicone fluid (MS 1107) in ethyl acetate.

9. Trypan blue solution.

Trypan blue (Sigma, Chemical Company Ltd, Poole, England)

2 %(w/v) in water.

10. X5 saline.

X5 saline is 4.25 % NaCl (w/v) in water.

11. Low calcium medium.

Quantities for 1 litre	
NaCl	8 g
KCl	0.4 g
D-glucose	1 g
Hepes	2.38 g
$CaCl_2$ (0.75mM)	0.11 g
EGTA (1mM)	0.38 g

pH was adjusted to 7.4

2.1.4. Disaggregation agents.

2.1.4-1. Versene (EDTA) solution: A phosphate-buffered balanced salt solution containing 0.53 mM ethylene diamine tetra acetate (sodium-EDTA: versene) for use in mixture with trypsin as a disaggregating agent of cell monolayers.

Ingredients for versene.

Quantities for 5 litres.	
NaCl	40 g
KCl	1 g
Na_2HPO_4 (anhydrous)	5.75 g
KH_2PO_4 (anhydrous)	1 g
EDTA (disodium salt)	1 g
0.5 % Phenol red	10 ml

Dispensed in 20 ml aliquots, autoclaved for 20 mins at 121°C and stored at 4°C.

2.1.4-2. Trypsin solution: Phosphate buffered balanced salt solution containing the digestive enzyme trypsin at a final concentration of 0.25% (w/v) of 1250-1500 BAEE unit activity per ml

(Gibco Ltd, Paisley Scotland).

2.1.5. Reagents.

1. Fatty acids and acyls-CoA derivatives.

Arachidonic acid (99%), linoleic acid, arachidonoyl -CoA (90%), linolenoyl-CoA (90%), linoleoyl-CoA (90%), oleoyl-CoA (95%) and stearoyl-CoA (96%) (Sigma, Chemical Company Ltd, Poole, England). ^{14}C-(oleoyl-CoA and arachidonoyl-CoA) with specific activity 52.2 mCurie/mmol and 50 mcurie/ mmol respectively (Amersham International).

2. Arachidonic acid derivatives.

Prostaglandins (PGs):E_1; E_2; I_2 (prostacyclin), Leukotriene B_4 (Sigma, Chemical Company Ltd, Poole, England).

3. Others.

^3H-inositol tris-phosphate(IP_3) (Amersham International) with a specific activity 1 curie / mmol (Becquerel/mmol), cold IP_3, Coenzyme A, adenosine triphosphate, phorbol myristate acetate, dextran-500, bromophenacyl bromide, mepacrine (Sigma Chemical Company Ltd, Poole England), diacylglycerol kinase inhibitors (Janssen, Lincoln Road, Cressex Industrial Estate High Wycombe Bucks HP12 3XJ), ionomycin (Calbiochem, PO box 12087, San Diego California. USA) and polyethylene glycol (BDH chemical Ltd. Poole england).

Fibronectin preparation. Fibronectin was used for coating polystyrene tissue culture dishes to improve cell adhesion and to investigate fibronectin-mediated adhesion. It was isolated from bovine serum on a gelatin-sepharose column using the method of Engval & Ruoslahti 1977.

2.2.Methods.

2.2.1. Cell culture.

Baby hamster kidney (BHK 21) and mouse pulmonary capillary endothelial cell line (B10.D2) were obtained from departmental stock frozen at -70°C in a mixture of foetal calf serum and dimethyl sulfoxide (DMSO).

To start the culture, a 1.8ml plastic vial containing 1 ml of $5x10^6$ cells was thawed out quickly and the content was transferred to a 20 ml glass universal containing fresh culture medium. Cells were then aspirated, spun at 1500 rpm for 5 minutes, resuspended in fresh medium and added in a total volume of 15 ml to 75 cm^2 tissue culture flasks. Cells were then left for few days to recover before being subcultured. Cells were grown attached to tissue culture flasks surface in bicarbonate buffered eagles medium (ECT) in 5% CO_2 atmosphere (BHK) or 3% serum Ham's F10 nutrient mixture medium (B10.D2) at 37°C.

Cells were subcultured by the following standard method when they reached confluency (approximately. 0.25-0.3 x 10^6 cells/cm^2) and the medium became acidic. The culture medium was removed and the monolayer washed twice with approximately 10 ml Hepes saline.

5 ml of trypsin/versene (1/4) were added and the cells were incubated for a period of 5 mins at 37°C. After this period the cells start detaching from the flask surface. Tryptic activity was then stopped by addition of 5 ml fresh ECT or 3% serum Ham's F10 to stop any further degradation which might damage the cell surface. The cell suspension was collected in sterile glass universals, aspirated with a pasteur pipette, counted on a haemocytometer, spun at 1500 rpm, resuspended in fresh medium, set at the required density and recultured in new 75 cm^2 tissue culture bottles.

The number of cells added to each culture bottle is dependent on the time at which the experiment being done and the number of cells required at that time.

2.2.2. Preparation of cells.

2.2.2-1. Preparation of BHK and B10.D2 cell suspensions.

Cells were trypsinised to provide cell suspension by adding 5 ml 1/4 trypsin-versene and then incubated for 5 minutes at 37°C till all the cells detached. Trypsin activity was stopped by addition of either 5 ml 3% serum Ham's F10 (in those experiments in which cells were tested for their adhesion in the presence of serum) or 25 µg/ml leupeptin (in experiments designed to test cell adhesion in serum free conditions). Then, cells were aspirated, spun at 1500 rpm, resuspended in the appropriate medium and set at the required concentration.

2.2.2-2. Preparation of PMN leukocyte suspension.

Human neutrophils were isolated from heparinized (10 U/ml) venous blood by means of Dextran sedimentation and Ficoll-Hypaque density gradient centrifugation.

Briefly, 20 ml of the whole blood was allowed to settle in plastic universal with heparin (10 U/ml blood) and 2 ml Dextran 110 for 60 mins at 37°C. The supernatant was gently added to an equal volume of Ficoll-Hypaque, and centrifuged at 1200 rpm for 30 mins. The pellet was washed in Hepes saline, and contaminating erythrocytes were removed by hypotonic lysis.

The cells were then resuspended in the appropriate medium.

2.2.2-3. Cell counting (number of cells/ml).

Cells were counted using a haemocytometer (Fudis-Rasenyhal) The total volume occupied by 8 squares is 10^{-4} ml from which the number of cells /ml can be calculated.

cells/ml = number in 8 squares x 10^4. The number of cells in 16 squares was counted and the mean obtained.

2.2.2-4. Cell viability test.

Cell viability was determined by staining the cell populations with trypan blue (Mishell & Shiigi, 1980). Viable cells exclude the dye, while non-viable take it up.

On the day of the experiment, 4 parts of 0.2% trypan blue were mixed with 1 part of x5 saline. To one part of the trypan blue-saline solution 1 part of the cell suspension ($2x10^6$/ml) was added. The cells were loaded into lined haemocytometer and the number of unstained (viable) and stained(dead) cells were counted separately in 8 squares.

$$\% \text{ viable cells} = \frac{\text{No.of viable cells}}{\text{No.of viable + non viable}} \times 100$$

2.2.3. Cell adhesion assay.

2.2.3-1. Cell-substratum adhesion assay.

i. Adhesion to polystyrene tissue culture dishes.

3 ml of a suspension of $0.2x10^6$/ml BHK cells were added to each polystyrene tissue culture grade dishes and incubated for 20 mins at 37°C. The cells that had not adhered during this period were washed off with Hepes saline.

The cells adherent to culture dishes were fixed in formal saline (20 mins), stained in 0.1% Kenacid blue (20 mins) and washed with water.

The number of cells adherent in each of 30 standard counting areas from a triplicate dishes (unless otherwise stated) was then counted using microscope to detect the cells and either a Quantimet 720 image analyzing computer (Cambridge Instruments Royston) or an image analyzing system based on a video camera, a Matrox 512 frame grubber (Matrox electronic systems Ltd. Quebec, Canada) and Semper 6 software (Synoptics, Cambridge) running on a Compaq 386 computer to count the cells.

The results can be expressed in terms of the number of cells adhering per unit area, per cm^2, percent from the control samples

or as a percentage from the following equation.

$$\% \text{ of cells adhering} = \frac{\text{cells adhering/unit area} \times 100}{T \times \frac{a}{A}}$$

T = the total number of cells in the dish .

A = the area of the dish(cm^2).

a = the counting area under the microscope (unit area).

ii. Adhesion to fibronectin coated polystyrene tissue culture dishes.

Fibronectin was first diluted to a final concentration of 25 µg/ml, then, 3 ml was added to each polystyrene tissue culture dish and allowed to adsorb on its surface for 30 mins at room temperature. The medium was then removed, unadsorbed fibronectin was washed off gently with Hepes saline and samples were added the same as mentioned above.

2.2.3-2. Cell-cell adhesion assay.

Aggregation studies were performed as described by Curtis, (1973). $4x10^6$ cells in 4 ml volume were shaken horizontally on a shaker in 10 ml siliconized conical flasks at 37°C with a constant speed of 75 ipm.

Samples were removed at regular intervals and the total number of cells singly or in aggregate in each of the 20 standard counting areas from duplicate samples were counted on an unlined haemocytometer using phase contrast microscopy to detect the cells and either of the image analysis systems described above.

The data is plotted as incubation time versus logarithm of the number of cells of all classes counted at time (t) divided by the number of cells at the starting time (t_0) according to Swift and Friedlander equation which was justified for aggregation studies by

Curtis, (1967); (1973).

2.2.3-3. Flow chamber adhesion assay.

Flow chamber assays were performed as described by Doroszewski et al., 1977 and Forrester and Lackie (1984). The flow chamber was constructed between two microscope slides. The two slides were separated by a gasket of Nescofilm (Nippon Shoji Kaisha Ltd, Japan) approximately 150 μm thick in which a channel of 4 mm wide and 40 mm long had been cut. The chamber was held in place by a metal and perspex clamping device with inlet and outlet ports that coincide with 4 mm holes drilled in the upper microscope slide.

A suspension of 2 x 10^6 /ml BHK cells were perfused through the chamber with a mechanical syringe driver at known flow rate(1.02 ml/hour).

The whole flow chamber was placed on the stage of an inverted Leitz microscope, under temperature of (37°C). Cells adhering to the lower surface of the flow channel were viewed through a monochrome video-camera/monitor system, which was connected to a time-lapse video-recorder with a time-date generator. The number of cells adherent at different times can be counted directly or by replaying the video tape. Results are expressed in terms of number of cells adherent per unit area of 3.38×10^{-3} cm^2.

2.2.4. Spreading assay.

The spreading assay was performed as described by Curtis and McMurray (1986). Briefly: cells adherent to poly styrene tissue culture grade dishes after 20 mins incubation (unless otherwise indicated) were fixed with formal saline, stained with kenacid blue and washed with water as described in the adhesion assay. Then, cells were visualised using x10 objective (unless otherwise stated) and the area of each cell was detected and measured using either of the image analysing systems described in section (2.2.3-1).

Approximately 200-500 cells were measured for each experimental condition in at least three replicate polystyrene tissue culture

dishes. The mean spreading area per cell is expressed as pixel or μm^2.

2.2.5. Testing the shearing force effect on adherent cells during the washing process of unattached cells.

Cells are usually incubated for a period of time, then non-adherent cells are washed off with Hepes saline. Approximately 3 ml are added carefully during the wash to each dish and shaken gently to remove only unattached cells.

During this process cells are a subject to a shearing force which is not absolutely accurate from one sample to another since the washing is manually operated.

Accordingly, it is important to demonstrate whether changes in the shearing force during the wash may also affect adherent cells.

Test. Cells were prepared in serum free medium as described before, set at $0.2x10^6$/ml and kept on ice.

3 ml of $0.2x10^6$ /ml cells were added to each of 8 polystyrene tissue culture dishes, incubated for 20 mins at 37°C and then the cells were washed with hepes saline.

The shearing force applied on the cell during the wash was deliberately increased in this case as follow:

1- The addition force of Hepes saline solution to the dish was increased gradually from the first sample to the last by an increment in the distance between the bottle of hepes saline and the dish surface.

2- The horizontally shaking force during the wash was also increased in consistent manner by increasing the hand movement (manuel shaking).

Cells were then fixed and counted. The results obtained does not show any significant difference between the samples mean as judged by t-test analysis.

The conclusion is that, varying the shearing force to a certain

degree has no significant effect on the attachment of already adherent cells and that is reasonable to use the fully crude method employed. This implies that the cells are attached and not easily removed.

2.2.6. The two-phase polymer gradient.

A two-phase polymer gradient of polyethylene glycol (PEG) and dextran 500, used for plasma membrane isolation, was prepared according to the method described by Evans (1987).

1. Stock solutions:

20% (w/w) dextran-500 (Sigma, Chemical Company Ltd, Poole, England) and 30% polyethylene glycol (BDH Chemical Ltd. Poole england) in distilled water. Both solutions were prepared by layering 200g dextran-500 or 300g PEG on 1 litre distilled water and heating with gentle stirring till dissolved.

2. 200g of 20% dextran and 103g of 30% PEG stock solutions were mixed by repeated inversion in separatory funnel with 33 ml of 0.2M phosphate buffer (Na-salts) at pH. 6.5 and 179 ml distilled water. The mixture was then allowed to settle at 4°C for 24 hours. After this period the two phases were collected separately as top PEG-phase and bottom dextran-phase (Fig.2.1).

2.2.7. Radioactive labelling of plasma membrane lipids.

$10x10^6$ Baby Hamster Kidney fibroblast were seeded in glass roller bottles (2.5 litre) of approximately 750 cm^2 lateral surface and were grown for 3 days in 5% CO_2 atmosphere at 37°C in bicarbonate buffered eagles medium (ECT) medium.

BHK cells were removed from the bottles by treatment for 5 mins with trypsin/versene solution (section2.1.4). Tryptic activity was stopped by adding either 3% serum Ham's F-10 or 25 µg/ml leupeptin depending on the design of the experiment. Cells were then washed, counted and resuspended in 1 ml fresh serum or serum free medium. Approximately, $35-40x10^7$ cells were used in this type of experiment.

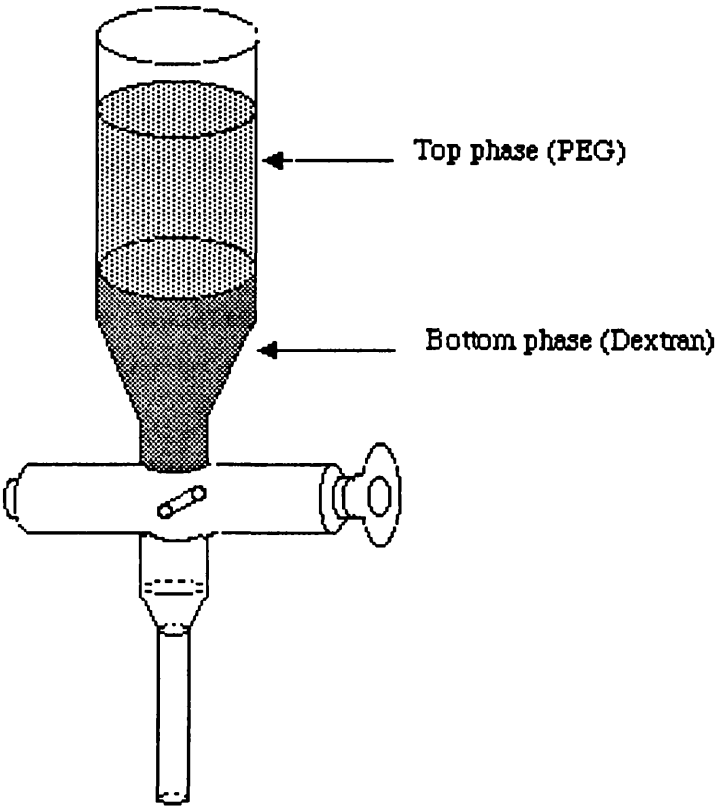


Fig.2.1. The two phase-polymer formation.
200g of 20% dextran and 103g of 30% PEG stock solutions were mixed by repeated inversion in separatory funnel with 33 mls of 0.2 M phosphate buffer (Na-salts) at pH. 6.5 and 179 ml distilled water. The mixture was then allowed to settle at 4°C for 24 hours. After this period the two phases were collected separately as top PEG-phase and bottom dextran-phase.

The 1 ml cells was mixed with 9 ml incorporation medium which consists of 12.5 μM ATP and $5x10^{-6}$ M ^{14}C-oleoyl-CoA at specific activity of 52.2 mCi/mmol and incubated for 20 mins at 37°C. The cells were then washed first with $5x10^{-6}$ M unlabeled oleoyl-CoA in serum free Ham's F-10 and then with 0.15 M NaCl, 30 mM $NaHCO_3$, pH.7.0. The cells were then resuspended in 30 mM $NaHCO_3$ and homogenized for plasma membrane isolation (see bellow).

2.2.8. Plasma membrane isolation.

Plasmalemmal fractions were isolated using dextran-PEG aqueous two-phase polymer system according to the method described by Gruber et el., 1984 and Evans 1987.

The cell suspension was prepared as above, washed twice with 0.15 M NaCl, 30 mM $NaHCO_3$, pH.7.0 and resuspended in 30 mM $NaHCO_3$ pH.7.0. The suspension was gently shaken for 15 mins at room temperature then cooled to 0°C and homogenized in tight fitting homogenizer (Baird & Tatlock (London) Ltd.). The degree of cell breakage was monitored by phase-contrast microscopy. The homogenate was then fractioned according to the flow chart illustration in Fig.(2.2).

Briefly, the 3 crude membrane pellets from 2000g and 12000g centrifugation were vortexed for 20 seconds with 10 ml top-phase (PEG) and mixed with 10 ml of Dextran-500 (bottom-phase). The mixture was placed in MSE high speed rotor (MSE Ltd, London) and spun at 12000g for 10 mins. On completion of this centrifugation the plasmalemmal fractions are found at the interface. The plasma membranes were collected and the two phase-system was remixed again in another tube, spun at 12000g for 15 mins and the material at the interface was also collected. The purified plasma membrane fractions were then diluted with 30 mM $NaHCO_3$ pH.7.0 to a point where the two-phase system no longer form, then washed and resuspended in small volume of 30 mM $NaHCO_3$ for further analysis.

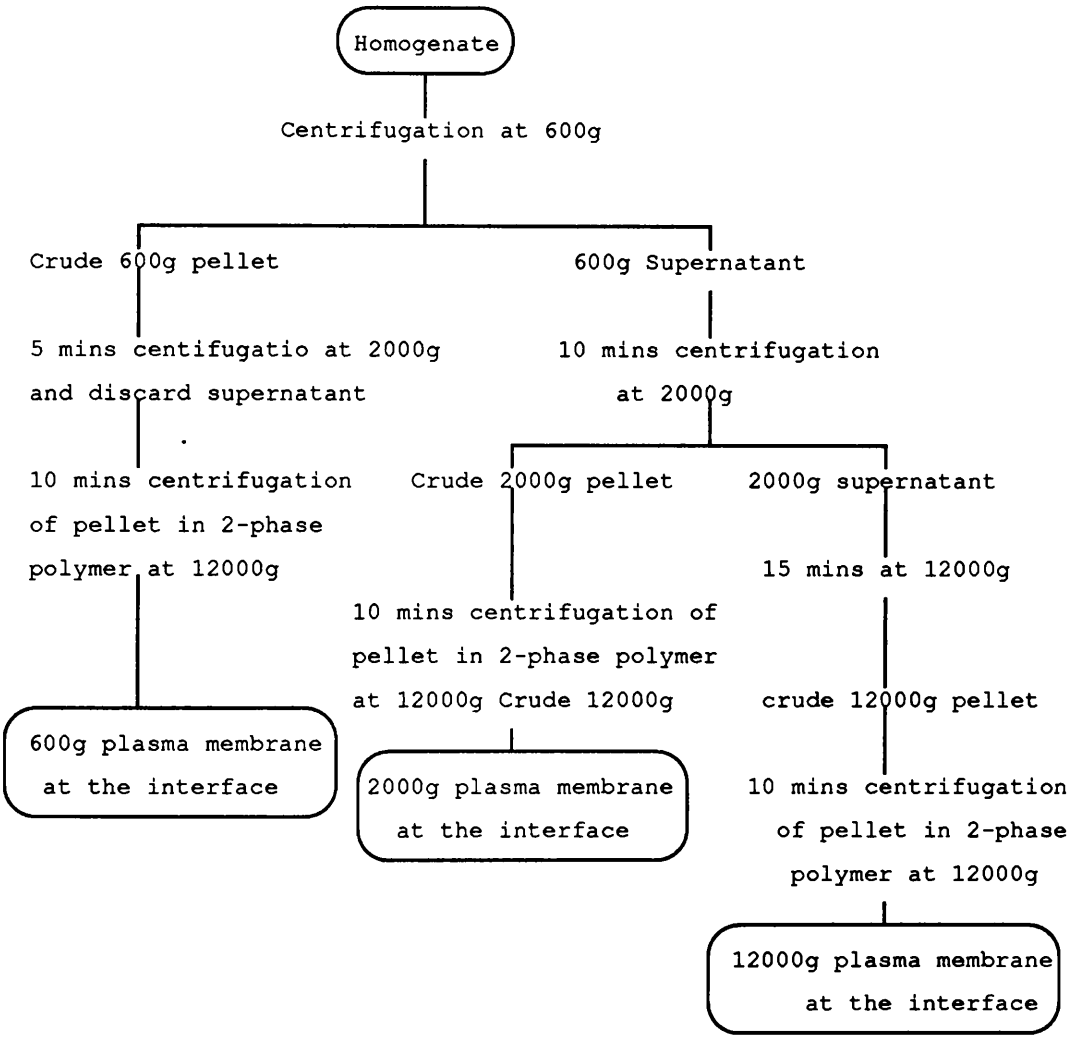


Fig.2.2. Illustration of the procedure of plasma membrane isolation from a homogenate of BHK cells

2.2.9. Extraction of plasma membrane lipids.

Plasma membrane phospholipids were extracted using chloroform /methanol (2/1) as follow:

1. In glass tubes an appropriate volume of chloroform/methanol was added to the purified membrane pellet and mixed very well until single phase is produced.

2. The tubes were covered and allowed to stand for at least 30 mins, and then the denaturated proteins were removed by centrifugation.

3. One-fifth the volume of 0.05 M $CaCl_2$ was added to the extract and mixed using a vortex mixer. This was centrifuged in a bench centrifuge to separate the two phases.

4. The upper phase was removed carefully and the lower phase which consists mainly of chloroform containing the lipids was recovered.

5. The chloroform was evaporated, the lipid yield was calculated and resuspended in the appropriate volume of chloroform.

2.2.10. Separation and identification of plasma membrane lipids.

Thin layer chromatography (TLC) system was used to separate and identify lipids that have been extracted with chloroform.

Silica gel on plastic as well as on glass plates was used. The solvent system consists of:

Chloroform (170), methanol (30), acetic acid (20) and water (7). Using this system, the separation in order of increasing the R_f value is phosphatidyl inositol, sulpholipids, lecithin, digalactosyl-diglyceride,phosphatidylglycerol, phosphatidylethanolamine, monogalactosyl diglyceride.

Appropriate standards were run in parallel using the same eluant and their R_f values were calculated which provided some degree of identification of the spots obtained from the crude lipid extract. Separated lipids were then detected non-destructively by staining with iodine vapor, scraped off, added to scintillation fluid, and

radioactivity was counted in a Beckman CPM 200 scintillation counter.

2.2.11. Inositol trisphosphate (IP_3) binding experiment.

The experiment was carried out according to the method described by Levitzki (1980) with modification.

In brief, BHK cells were prepared in serum free conditions as mentioned above in this chapter and set at concentration of $1x10^7$/ml. ^{3}-H-IP_3 (specific activity 1Ci/mmol) was then added to $3x10^7$ cells at a final concentration between $8.3x10^{-8}$ and $1.6x10^{-10}$ M, and the mixture was incubated for 15 mins at 37°C. After this period cells were washed with serum-free medium to remove the free IP_3. The radioactivity in cells and the wash (free) was counted separately in Beckman CPM 200 scintillation counter.

2.2.12. Statistical tests.

Student t-test was used to evaluate the significance of differences between the population means obtained by random sampling from a normal or approximately normal distribution.

These symbols (*, **, ***) are used to demonstrate the degree of significance.

* = $P<0.05$.

** = $P< 0.01$

*** = $P< 0.001$

Chapter III
Results

III.Results.

The results are divided into two main sections:

3.1, membrane phospholipid modulation and cell adhesion.

3.2, cell activation and adhesion, which include the effect of arachidonic acid metabolites, phospholipase A_2 inhibitors and agents that affect calcium/protein kinase C pathways which may affect cell adhesion.

3.1. Membrane phospholipid modulation and BHK cell adhesion.

It has been shown that the lipid composition of cells can be modulated by incorporating selected fatty acids supplied exogenously in the free form into phospholipids of plasma membrane by taking advantage of its acyl transferase and the reacylation system. Such alteration in the composition of lipids have been also shown to affect many cell functions including growth, differentiation and cell adhesion.

The general experimental design in this section has been to investigate the effect of various fatty acids, supplied as acyl-CoAs, that differ in the degree of saturation and chain length on the adhesion, spreading and viability of BHK fibroblast cells and the mechanisms of action based on cell-substrate interaction.

In many ways the experimental protocols used by Curtis et al., (1975), (1983) and Hoover et al., (1977), were applied in the present work but in addition, because of the finding by Curtis and McMurray (1986) on the effect of serum proteins on attachment, additional measurements of adhesion were made in the absence of serum. In such experiments leupeptin (rather than serum) was used to inhibit trypsin. These matters are discussed more fully in the next paragraph.

1. Serum is usually used to stop tryptic activity after cells have been trypsinised. Curtis et al., 1986 showed that even traces of serum could adsorb to cells, so they then behave as though they were adhering in the presence of serum. Adhesive interactions appear to be modified in serum, thus, leupeptin is probably the best agent for stopping tryptic activity without introducing serum or serum like effects. Leupeptin is the name of a group of *Streptomyces* products that inhibit proteases and includes acetyl- or propionyl-l-leucyl-l-leucyl-argininal and their analogues, a peptide derivative in which COOH terminal is changed to a terminal aldehyde (Kondo et al., 1969). These peptide aldehydes are highly selective and potent serine and cysteine-protease inhibitors (see Frankfater & Kuppy 1981 for mechanism of action).

2. Adhesion was measured by incubating the cells for 20 minutes on polystyrene tissue culture (TC) dishes, then the number of adherent cells was counted using a video camera microscope connected to a Quantimet image analysing computer or a Matrox digitizer feeding digitized data to an IBM or Compaq 386 and analysed by a Semper6 program (see methods). This method gives the opportunity for the cells to settle on a tissue culture grade dish surface for a specific time, then unattached cells can be removed readily and the adherent are counted accurately.

3. Fatty acyl-Coenzyme A was used instead of free fatty acid to avoid the effects of ethanol employed in other work to solubilise the fatty acids, because of the greater solubility in water and low toxicity of these fatty acyl-coA. However, in comparison experiments fatty acids were used as a free acid form and dissolved in a minimum concentration of ethanol at final concentration of less 1%.

4. Adenosine triphosphate (ATP) at concentration of 1.25×10^{-5} M

was added along with acyl-CoA as it was already demonstrated to be required for the reacylation reaction. ATP levels are critical, possibly because high concentrations chelate divalent cations from the medium.

5. Phospholipase A_2 is a phosphatide 2-acyl hydrolase which has many functions among them, the hydrolysis of the central ester linkage in phospholipids in presence of calcium (Stotboom et al., 1982) and the release of arachidonic acid from arachidonyl phosphatidyl lipids (McKean et al., 1981).

The idea behind the pretreatment of cells with ethylenedianine tetraacetic acid (EDTA), is in order to inhibit any phospholipase A_2-induced membrane turnover before measurement began.

3.1.1.Saturated fatty acyl-CoA effect on BHK cell adhesion.

3.1.1-1. Stearoyl-CoA. Has a saturated hydrocarbon chain (18:0) in which the carboxyl group interacts with the free sulfahydryl group on the end of the Coenzyme A and forms a thioester bond.

The effect on cell adhesion of this 18 carbon chain fatty acid was examined first in serum-free medium, and then in presence of serum.

i. Adhesion in serum-free medium.

BHK cell suspensions were prepared as described in Methods. Briefly, cells were trypsinised using a trypsin/versene mixture and allowed to stand at 37°C for few minutes till cells start to detach. Leupeptin (25µg/ml) was used to stop tryptic activity in those experiments. The cells were washed, resuspended in fresh serum-free Hams F-10 medium and set at 0.2×10^6/ml. A concentration of 3.5 and 7 µM stearoyl-coA in presence of 1.25×10^{-5} M ATP was added to the cells in glass universals. 3 ml of 0.2×10^6 cells/ml were added to each of three replicate dishes, incubated for 20 mins at 37°C and then adherent cells were counted after removal of

non-adherent cells by washing with Hepes saline (HS). A significant ($P<0.001$) decrease in the adhesion was observed at both concentrations (3.5 and 7μM) in comparison with control samples (fig.3.1). The reduction in adhesion was approximately 32 and 75% of the control. However, BHK cells that have been first preincubated in 20 ml glass universals for 20 mins at 37°C in serum-free Ham's F-10 medium (Fig.3.2) or 2.5 mM EDTA (Fig.3.3) prior to the addition of 3.5 and 7 μM stearoyl-CoA and 12.5 μM ATP show a significant ($P<0.05$) recovery in their adhesion after 20 mins incubation at 37°C in polystyrene TC dishes. In fact there was a significant (*t-test*) enhancement of adhesion in comparison with the first experiment when 3.5μM was used. This recovery in cell adhesion might be related to the repair process during the preincubation period that might some how prevented the effect seen when the cells are exposed to stearoyl-CoA straight on after inhibition of trypsin activity.

In another experiment BHK cells were trypsinised, leupeptin used to stop tryptic activity, resuspended in serum-free medium and preincubated with 12.5 μM ATP and a range of stearoyl-CoA concentrations (1.75; 3.5; 7; 14; 28μM) in 20 ml glass universals for 20 mins at 37°C. Then, 3 ml $0.2x10^6$ cells/ml were added to each of three polystyrene TC dishes and incubated for 20 mins at 37°C in the continuous presence of stearoyl-CoA. A marked increase in adherence was observed at the following concentrations, 1.75, 3.5 and 7μM (Fig.3.4) in comparison with control samples ($P<0.001$), while a significant ($p<0.001$) decrease in adhesion was observed at higher concentrations (14, 28 μM) (fig.3.5).

In similar conditions, BHK cells were first preincubated for 20 mins at 37°C in 20 ml glass universals with 2.5 mM EDTA, in order to stop lipid turnover, 12.5 μM ATP and different concentrations

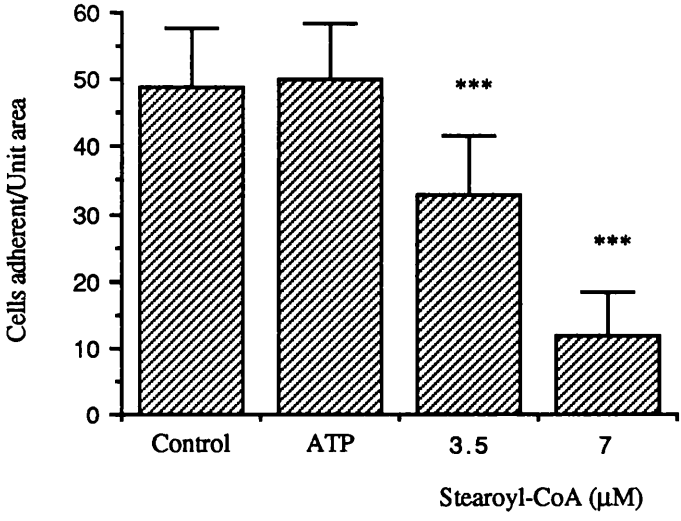


Fig.3.1. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene tissue culture (TC) dishes in serum-free Ham's F-10 medium. (No preincubation)

BHK cells were first trypsinised and 5 ml (25 μg/ml) leupeptin used to stop trypsin activity, washed, and then resuspended in fresh serum-free Ham's F-10 medium and set at $0.2x10^6$/ml. Stearoyl-CoA (3.5 & 7μM) and $1.25x10^{-5}$ M ATP were then added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out with Hepes saline and adherent cells were fixed with formal saline, stained with kenacid blue and then counted using either of the image analysing systems described in Methods. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars are one standard deviation.

*** Differ significantly from the control *(t-test), P< 0.001.*

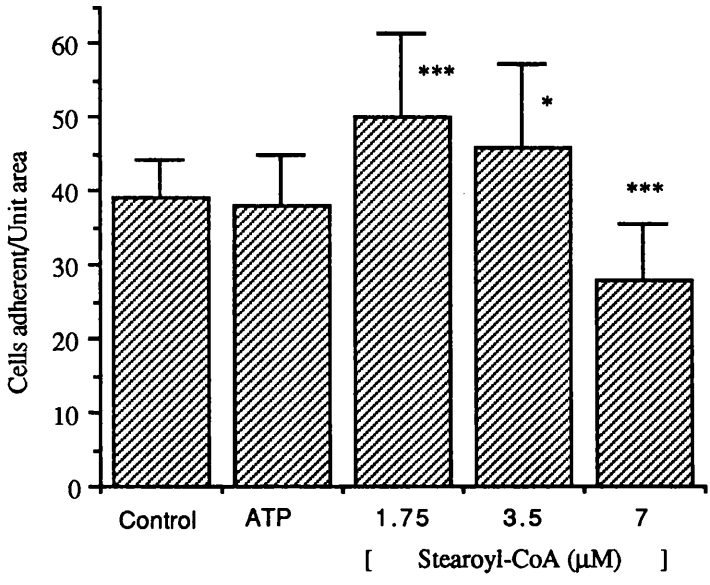


Fig.3.2. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium. (Preincubation in serum-free medium)

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals in serum-free medium for 20 mins at 37°C. Then, stearoyl-CoA and 1.25×10^{-5} M ATP were added to the cells and 3 ml (0.2×10^6/ml cells) were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

***, * Differ significantly from the control *(t-test), $P < 0.001$ and $P < 0.05$ respectively.*

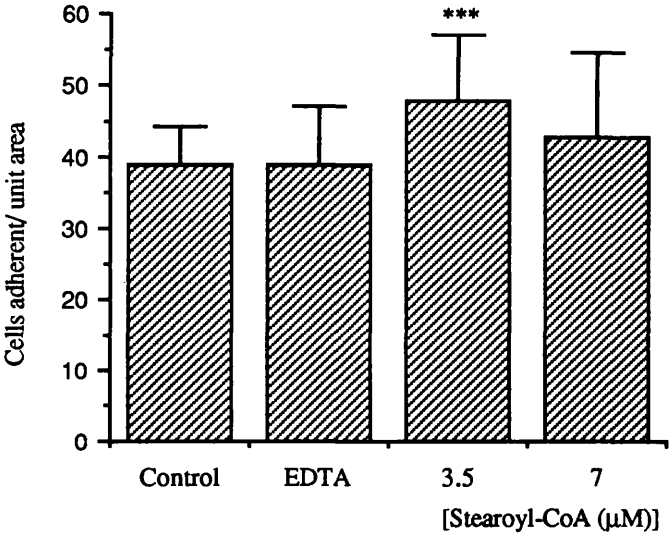


Fig.3.3. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium in presence of EDTA.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with 2.5 mM EDTA in serum-free medium for 20 mins at 37°C. Then, stearoyl-CoA and 1.25×10^{-5} M ATP were added to the cells and 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C in the continuous presence of EDTA. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), $P < 0.001$.*

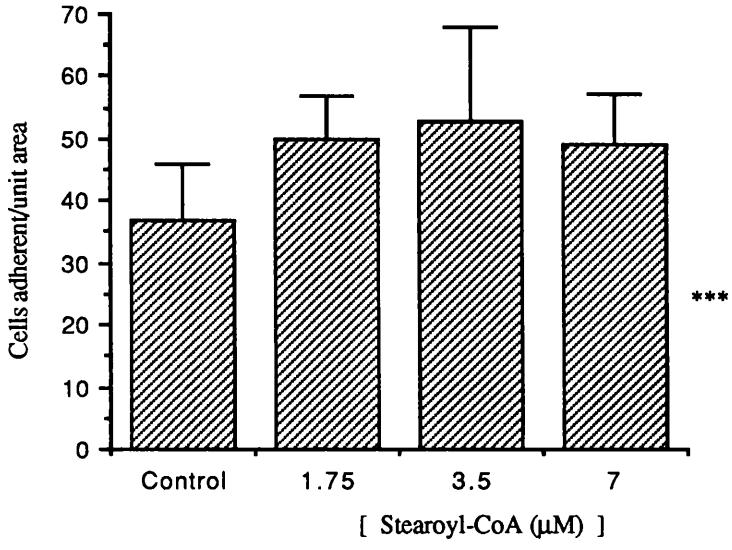


Fig.3.4. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

***All bars differ significantly from the control *(t-test), $P < 0.001$.*

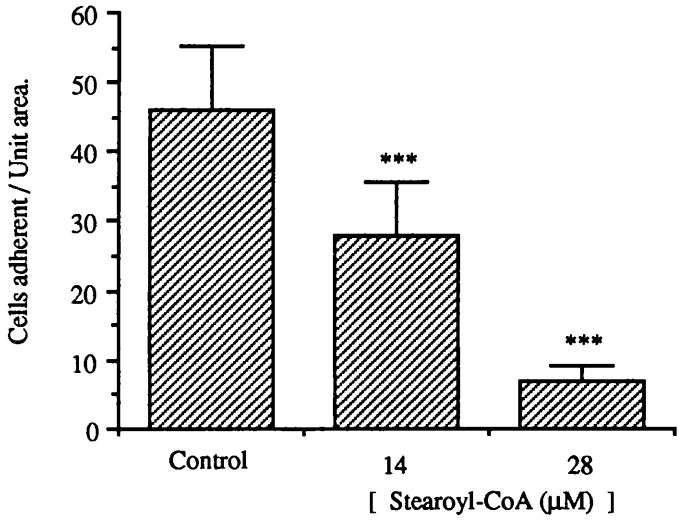


Fig.3.5. The effect of high concentrations of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), P< 0.001.*

of stearoyl-CoA. After this, the cells were added to polystyrene TC dishes for measurement of adhesion at 37°C in the continuous presence of stearoyl-CoA. A similar increase in BHK cell attachment was observed at concentration lower than 7μM, whereas, a significant ($P<0.001$) decrease at concentrations higher than that (Fig.3.6) and (plate.1). It is also clear from plate (1) that the spreading was affected under the above conditions.

ii. Adhesion in presence of serum.

I then sought to determine whether similar effects can be seen in presence of serum. In this experiments, 3% serum Ham's F-10 medium rather than leupeptin was used to stop trypsin activity. Cell adhesion under this conditions was not altered (Fig.3.7) and cells did not respond to any stearoyl-CoA concentration used, unlike those that have been resuspended in serum-free medium after leupeptin was used to stop trypsin activity.

It is clear from the above results (i,ii) that the presence of serum in the medium during the attachment process altered the effect of stearoyl-CoA on BHK cell adhesion.

Therefore, it was of importance to examine whether the method used to stop trypsin activity before the adhesion process lead to results different from the above. The effect of stearoyl-CoA on BHK cell adhesion was first examined in serum-free medium after trypsin activity was stopped with 3% serum-containing medium and secondly in serum-containing medium after trypsinization was stopped with leupeptin.

It was found (Fig.3.8) that the use of serum-containing medium to stop trypsin activity and measuring the adhesion in serum-free medium lead to results similar to those in which leupeptin was used

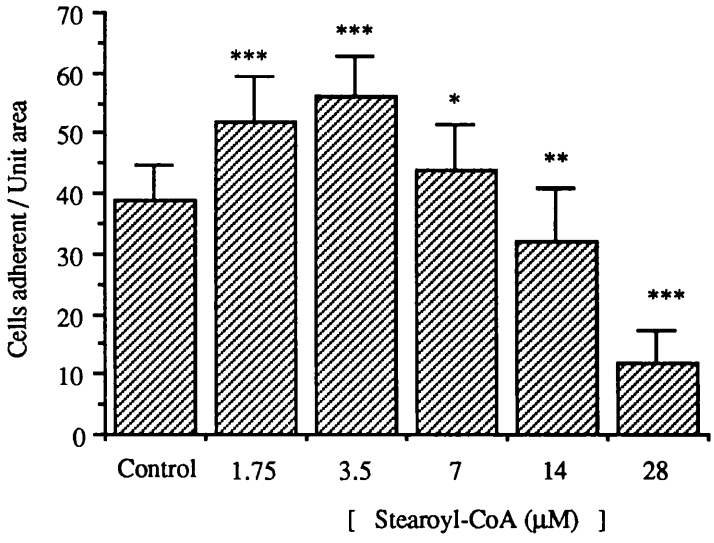
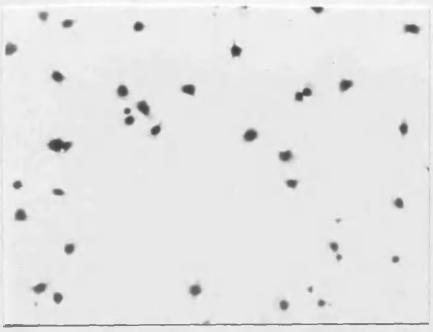


Fig.3.6. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium in presence of EDTA.

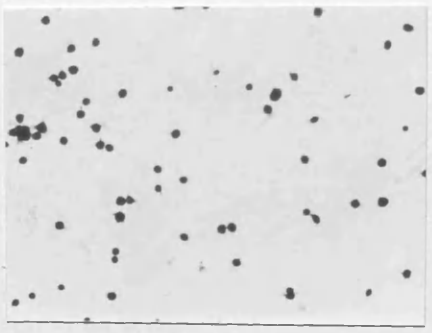
BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA, 1.25×10^{-5} M ATP and 2.5 mM EDTA in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C in the continuous presence of EDTA.. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

***, **, * Differ significantly from the control *(t-test), P< (0.001, 0.01 and 0.05 respectively)*.

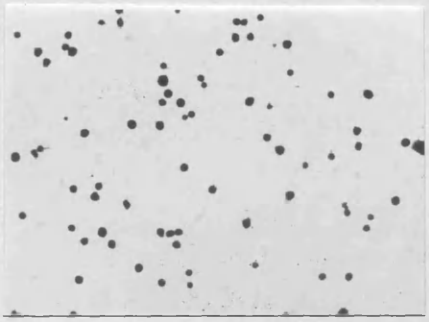
Plate.1. The effect of stearoyl-CoA on BHK cell adhesion and spreading in presence of EDTA in serum-free medium.
Cells were preincubated only in serum-free medium (control) (a), or with EDTA plus ATP plus [1.75 μM (b), 3.5 μM (c), 7 μM (d), 14 μM (e) or 28 μM stearoyl-CoA (f)] for 20 mins at 37°C, then plated into polystyrene tissue culture dishes. After 20 mins incubation at 37°C, attached cells were fixed with formal saline, stained with kenacid blue washed with water and then photographed.
Scale bar = 100 μM



a



b



c

d

e

f

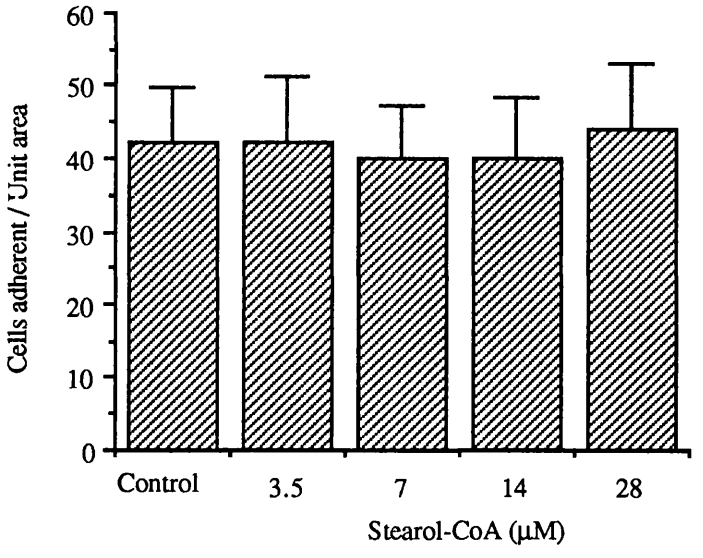


Fig.3.7. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

Cells were trypsinised, 3% serum Ham's F-10 was used to stop trypsin activity, span and resuspended in fresh 3% serum Ham's F-10. Cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA, and 1.25 x 10^{-5} M ATP in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

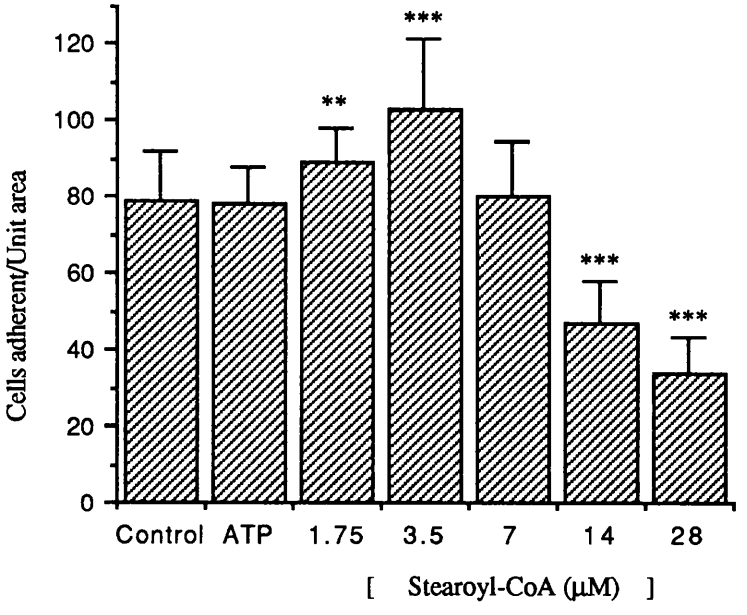


Fig.3.8. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in relation to the method of cell-suspension preparation.

Serum medium was used to stop trypsin activity and the adhesion was measured in serum-free medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, cells were added to polystyrene TC dishes and incubated for 20 mins at 37°C. Adhesion is expressed as number of cells adherent per unit area of 6.3×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

, * Differ significantly from the control (*t-test*), *P< (0.01 and 0.001 respectively)*.

to stop trypsin activity (i). A significant enhancement (*t-test*) of adhesion was seen at lower concentrations but a sharp decrease in the adhesion when higher concentrations were used.

When leupeptin was used to stop trypsin activity, the adhesion of stearoyl-CoA treated BHK cells in serum-containing medium was not affected at all (Fig.3.9). In other words, the presence of serum in the adhesion medium during the adhesion assay stopped the effect of stearoyl-CoA on BHK cell adhesion. However, the use of serum-free medium during the adhesion assay did not stop stearoyl-CoA effect on BHK cell adhesion. This might suggest that the stearoyl-CoA effect on BHK adhesion is altered by the presence of serum in the adhesion medium regardless of the method used to stop tryptic activity. A summary of the main effects of stearoyl-CoA on BHK cell adhesion in relation to the method used to stop trypsin activity is given in Fig.3.10.

iii. Cell spreading.

Since changes in adhesion often parallel changes in spreading, I also investigated whether similar effects on cell spreading can be seen under serum-free conditions.

BHK cells were preincubated for 20 mins at 37°C with different concentrations of stearoyl-CoA and ATP in serum-free conditions, added to polystyrene TC dishes and the spreading area was measured after 65 mins incubation at 37°C (Table.1). It is clear that the cell spreading decreased in a dose dependent manner, the higher the concentration the greater the effect (plate.2). A reduction in the cell spreading was also seen using 3.5μM stearoyl-CoA and different incubation times (Table.2). Cells were preincubated with ATP and Stearoyl-CoA for 20 mins, then plated into polystyrene TC dishes. Samples were removed at different times and cell spreading was measured. Cells show a significant decrease ($p<0.001$) in their

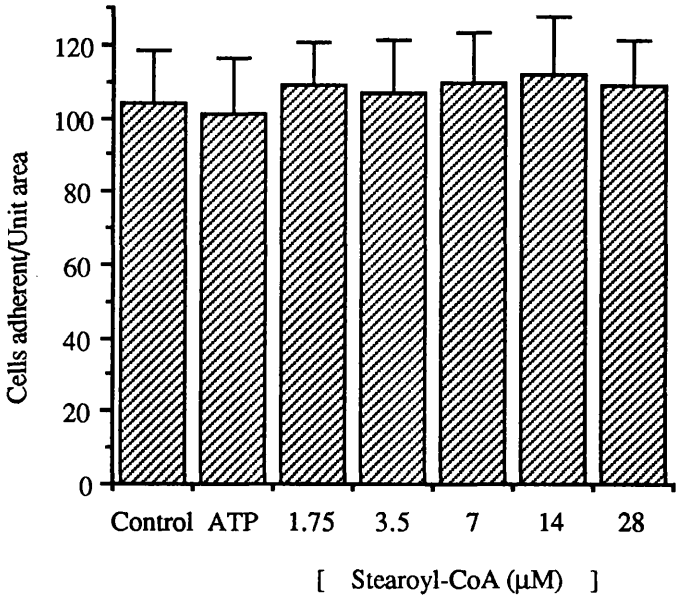


Fig.3.9. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in relation to the method of cell-suspension preparation.

Leupeptin was used to stop trypsin activity and the adhesion was measured in 3% serum-containing medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with stearoyl-CoA and 1.25×10^{-5} M ATP in 3% serum Ham's F-10 for 20 mins at 37°C. Cells were then added to polystyrene TC dishes and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 6.3×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

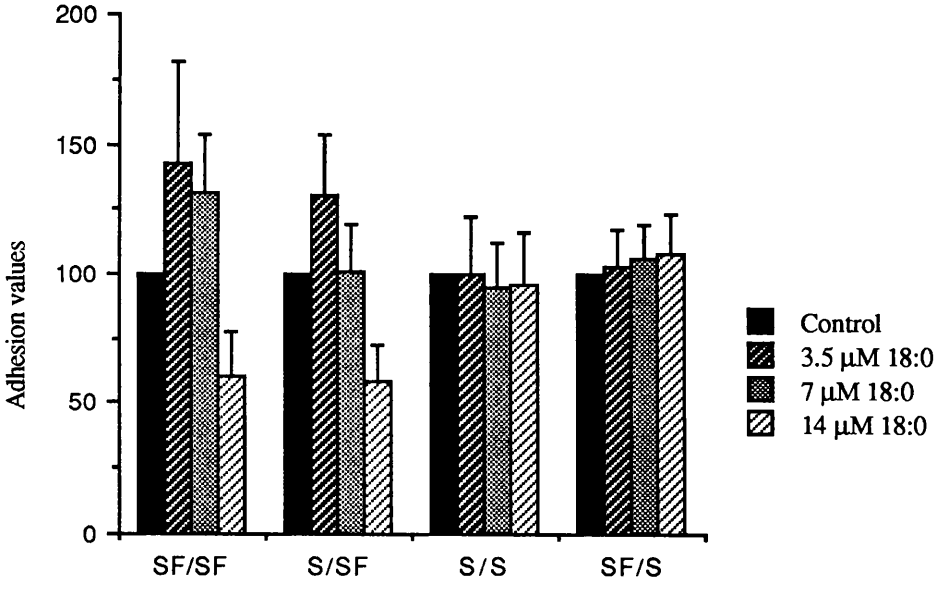


Fig.3.10. A summary of the effect of various concentrations of stearoyl-CoA on BHK cell adhesion in relation to the method used to stop tryptic activity.

SF/SF : Leupeptin used to stop trypsinization, and incubation and adhesion assay were performed in serum-free medium

S/SF : Serum medium used to stop trypsinization, and incubation and adhesion assay were carried out in serum-free conditions.

SF/S : Leupeptin used to stop trypsinization, and incubation and adhesion assay were performed in serum-containing medium.

SF/SF : Serum medium used to stop trypsinization, and incubation and adhesion assay were performed in serum-containing medium as well.

Table.1. The effect of stearoyl-CoA on BHK fibroblast cell spreading on polystyrene tissue culture (TC) dishes in serum-free Ham's F10 medium after 65 mins incubation at 37°C.

Stearoyl-CoA concentration (μM).	Mean spreading area/per cell (μm^2) ± Standard deviation
Control	593 ± 375.26
ATP	651 ± 362.18
1.75 + ATP	412 ± 250 (***)
3.50 + ATP	276 ± 200.94 (***)
7.00 + ATP	197 ± 144.27(***)

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with stearoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in serum-free Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 65 mins at 37°C. Unattached cells were then washed with Hepes saline (HS) and adherent cells were fixed with formal saline, stained with kenacid blue and the area of each adherent cell was measured using either of the image analysing systems described in Methods'section. Cells were visualized with a x10 objective and spreading is expressed as mean area per cell (μm_2) ± standard deviation. About 300 cells were measured for each experimental condition from three replicates.

***Differ significantly from the control *(t-test), P<0.001.*

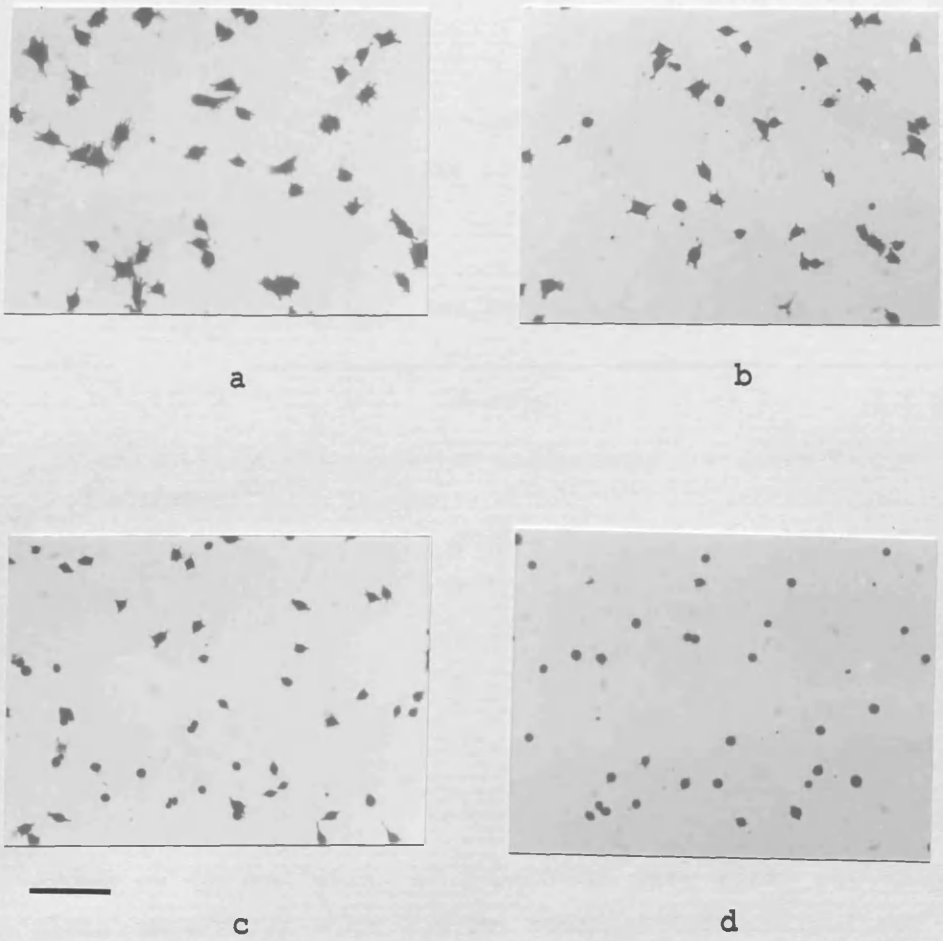


Plate. 2. The dose response effect of stearoyl-CoA on BHK cell spreading.

Cells were preincubated for 20 mins at 37°C, in serum-free medium (control) (a), or with ATP and [1.75 μM (b), 3.5 μM (c) or 7 μM stearoyl-CoA (d)]. Cells were then plated into polystyrene tissue culture dishes and incubated for 65 mins at 37°C. Adherent cells were then fixed with formal saline, stained with kenacid blue, washed with water and photographed.

Scale bar = 100 μm.

Table.2. Time course effect of stearoyl-CoA on BHK fibroblast cell spreading on polystyrene TC dishes in serum-free Ham's F10 medium.

	Mean spreading area/per cell (μm^2) ± Standard deviation	
	Control	3.5 μM (***)
Time (mins)		
20	399 ± 204.72	152 ± 42.43
30	389 ± 77.58	151 ± 30.45
40	566 ± 114.93	161 ± 25.98
50	543 ± 125.94	208 ± 85.99

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM stearoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in serum-free Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were washed and the area of each adherent cell was measured. Cells were visualized with 25x objective and spreading is expressed as mean area per cell (μm^2) ± standard deviation. More than 100 cells were measured for each experimental condition from three replicates.

***Differ significantly from the control *(t-test), P<0.001.*

spreading after 10 mins incubation and still showing approximately the same response after longer incubation periods. It should be noted however, that cells started recovering from stearoyl-CoA effect after 50 mins incubation and they showed a significant increase in their spreading compared with the spreading area after 20 mins (Table.2 and plate.3).

iv. Adhesion to fibronectin surfaces.

In a separate experiment, dishes precoated with fibronectin were used to assess the role of stearoyl-CoA in BHK cell adhesion to adsorbed proteins. 3 ml 25μg/ml fibronectin were added to each polystyrene TC dish and incubated for 30 mins. Cells were trypsinised, leupeptin being used to stop tryptic activity and were then resuspended in fresh serum-free medium. Cells were primarily preincubated for 20 mins at 37°C in serum-free medium, then, stearoyl-coA (3.5-28 μM) and ATP were added and the number of cells adherent to the fibronectin coated polystyrene TC dishes after 20 mins incubation at 37°C was counted. The adhesion was reduced significantly ($P<0.001$) at concentrations higher than 3.5 μM. 3.5 μM stearoyl-CoA and lower concentrations slightly ($P<0.01$) enhanced BHK cell adhesion (fig.3.11). Stearoyl-CoA therefore affected the adhesion of cells to uncoated substrate as well as to adsorbed proteins. This may indicate that cell adhesion to protein-coated surfaces dependent to a great extent on the fluidity of lipid bilayer that might affect receptor mobility. The effect could be explained also in terms of change in membrane electrodynamic forces (see Discussion).

It can be concluded that stearoyl-CoA affected the adhesion properties of BHK cells in serum-free condition to either clean or fibronectin precoated polystyrene TC dishes. Stearoyl-CoA had two

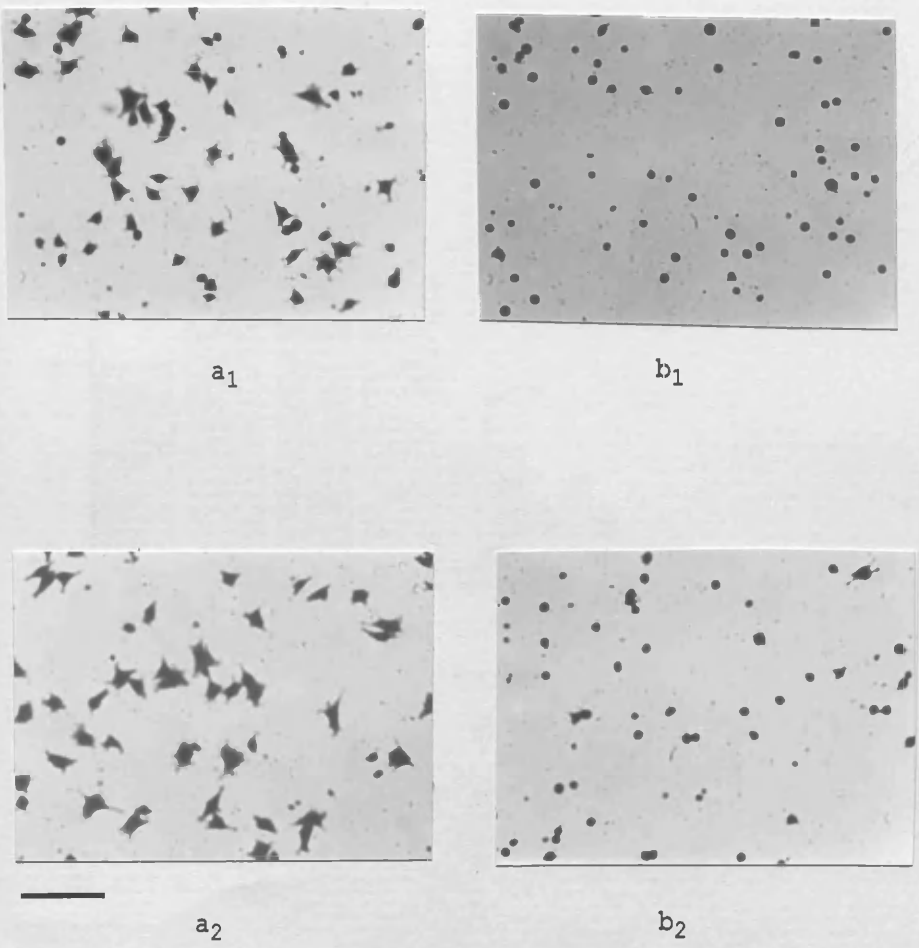


Plate.3. The time course effect of stearoyl-CoA on BHK cell spreading.

Cells were preincubated for 20 mins at 37°C, in serum-free medium (control) (a), or with ATP and 3.5 μM stearoyl-CoA (b). Cells were then plated into polystyrene tissue culture dishes and incubated at 37°C. Cells were fixed stained and photographed after 20 mins (a_1, b_1) or 50 mins (a_2, b_2).

Scale bar = 100 μm.

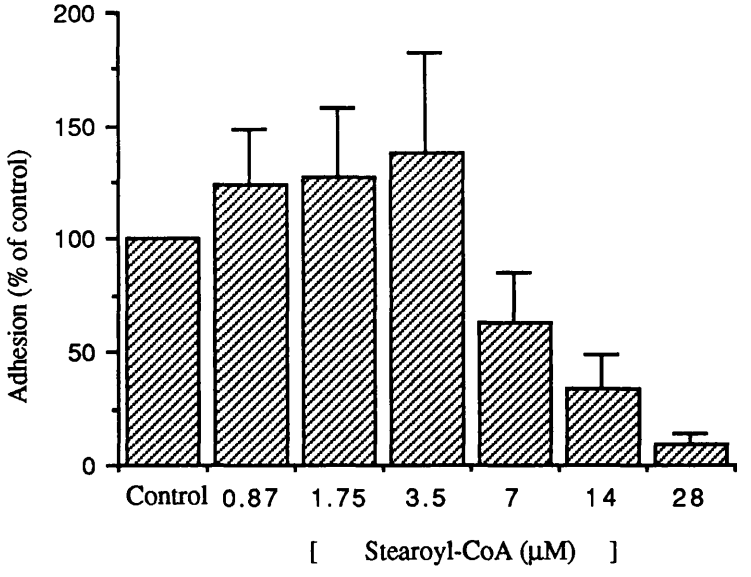


Fig.3.11. The effect of stearoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes coated with 25 μg/ml fibronectin in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated for 20 mins at 37°C in 20 ml glass universals in serum-free Ham's F-10. Then, stearoyl-CoA and 1.25×10^{-5} M ATP were added to the cells, and 3 ml (0.2×10^6/ml) cells were plated into each polystyrene TC dish precoated with 25 μg/ml fibronectin and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as percentage of the control samples.

Error bars are one standard deviation.

*** All bars differ significantly from the control *(t-test)*. *$P<0.001$*.

different effects on BHK cell adhesion, an enhancement at concentrations lower than 7μM and a reduction at concentrations higher than 7μM. However, no effect on the adhesion or spreading was found in presence of serum in the adhesion medium.

EDTA, did not modulate stearoyl-CoA (1.75-7μM) effect on BHK cell adhesion.

3.1.2. The effect of unsaturated fatty acyl-CoAs on BHK adhesion.

3.1.2-1. Arachidonoyl-coenzymeA. This acyl-CoA has a 20 hydrocarbon chain and 4 double bonds. Arachidonic acid is known to be released from arachidonyl-phospholipids under the action of phospholipase A_2. It is also, a precursor of prostaglandins, thromboxanes and leukotrienes. These metabolites are synthesised through two different pathways, cyclooxygenase, for PGs and TXs and lipoxygenase for LTs. They play a major role in many cellular functions including cell adhesion (O'Flaherty et al., 1979a; Whittle & Moncada 1983; McIntyre et al., 1986). Hence, it was of importance to test the effect of arachidonoyl-CoA on BHK cell adhesion.

i. Adhesion in serum free-medium.

Cells were first preincubated in 20 ml glass universals for 20 mins at 37°C in serum-free-medium with 2.5 μM EDTA and/or ATP and different concentrations (1.75-28μM) of arachidonoyl-CoA. Then, the cells were added to polystyrene TC dishes and cell adhesion was accessed after 20 mins incubation at 37°C. There was no clear effect on cell adhesion in both conditions(fig. 3.12; 3.13; 3.14 and 3.15).

ii. Adhesion in the presence of serum.

The effect of arachidonoyl-CoA on BHK cell adhesion under serum

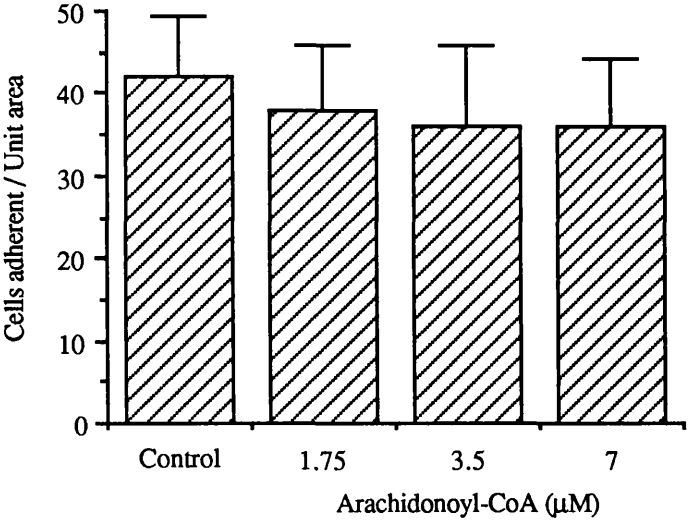


Fig.3.12. The effect of arachidonoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with arachidonoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

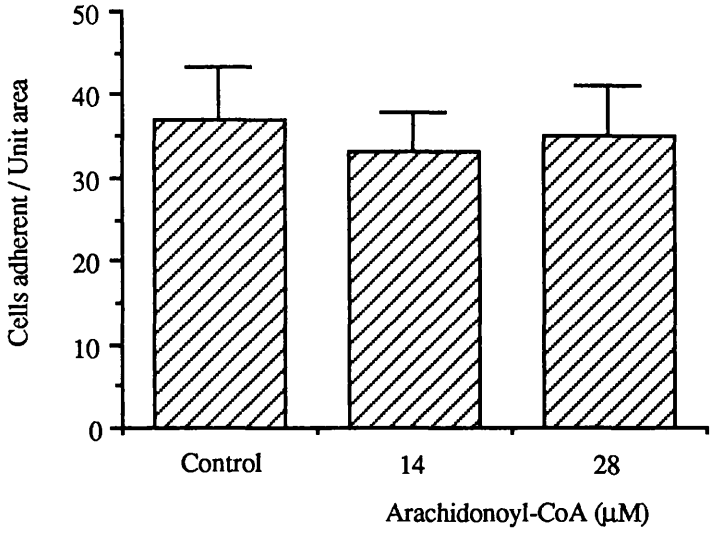


Fig.3.13. The effect of higher concentrations of arachidonoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in_serum-free Ham's F-10 medium.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with arachidonoyl-CoA and $1.25x10^{-5}$ M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

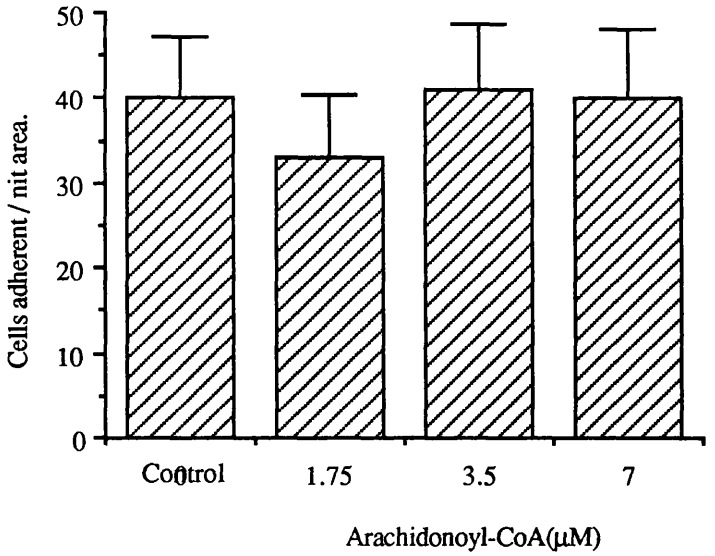


Fig.3.14. The effect of arachidonoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium in presence of EDTA.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with arachidonoyl-CoA, $1.25x10^{-5}$ M ATP and 2.5 mM EDTA in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C in the continuous presence of EDTA. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars are one standard deviation.

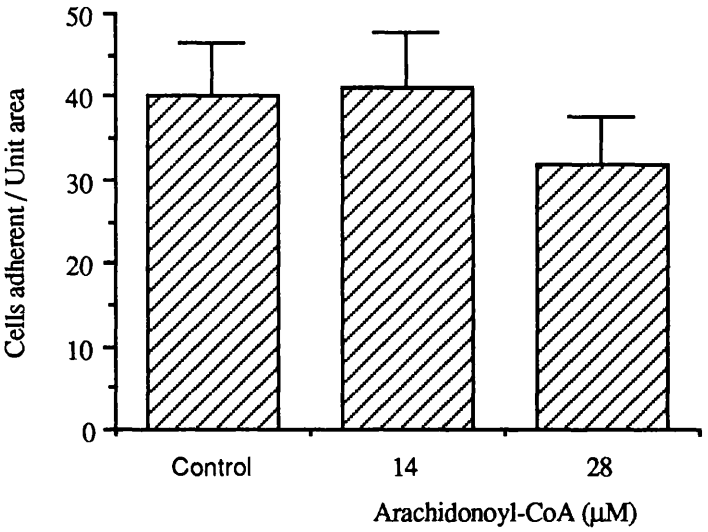


Fig.3.15. The effect of higher concentrations of arachidonoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium in presence of EDTA.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with arachidonoyl-CoA, $1.25x10^{-5}$ M ATP and 2.5 mM EDTA in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C in the continuous presence of EDTA. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

conditions was also investigated. Similarly cells did not produce any differences in their adhesion compared with the control samples (Fig.3.16). The spreading was not altered either in serum-free or in 3% serum Hams F-10 conditions.

3.1.2-2. Linolenoyl-CoA, is based on an unsaturated fatty acid having 18 carbon chain length with 3 double bonds.

Linolenoyl-CoA did not affect BHK adhesion either in presence of ATP (Fig. 3.17) or in presence of 12.5 μM ATP and 2.5 mM EDTA (Table.3) in serum-free conditions.

3.1.2-3. linoleoyl-CoA. The acid is an unsaturated having 18 carbon chain length and 2 double bonds.

Cells preincubated in glass universals for 20 mins at 37°C in serum-free Hams F-10 in a mixture with linoleoyl-CoA (1.75-28μM) and ATP (Fig.3.18) or ATP and 2.5 mM EDTA (Table.4), and then incubated for 20 mins at 37°C in tissue culture dishes, show a significant increase ($P<0.001$) in their adhesion at concentrations 1.75, 3.5 and 7 μM linoleoyl-CoA (Fig.3.18 & Table.4). Such results were not seen in 3% serum Ham's F-10 conditions using linolenoyl-CoA (18:3) or linoleoyl-CoA (18:2), see (Table.5 and 6). Similarly the spreading was not altered in both serum and serum-free conditions.

3.1.2-4.Oleoyl-CoA. This compound is based on 18 carbon atom chain length acid with one double bond. Its effect on cell adhesion as well as spreading was investigated.

i. Adhesion in serum-free medium.

In a dose-response experiment BHK cells were first pretreated in serum-free conditions for 20 mins at 37°C with oleoyl-CoA at concentrations ranging between 1.75 and 28 μM and 12.5 μM ATP.

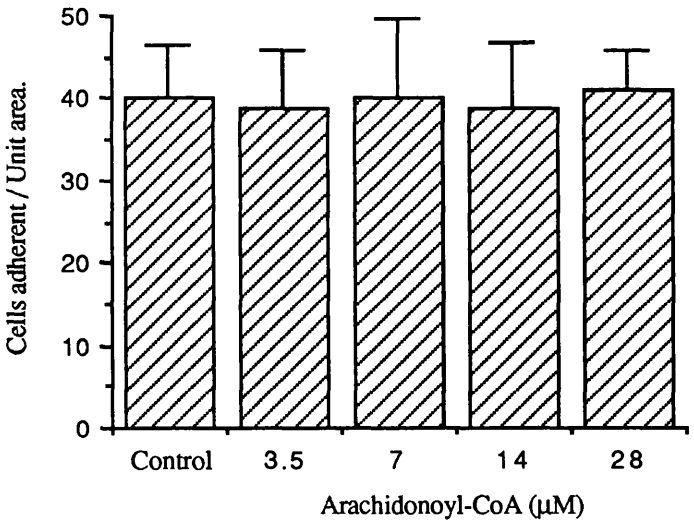


Fig.3.16. The effect of arachidonoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with arachidonoyl-CoA and 1.25×10^{-5} M ATP in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

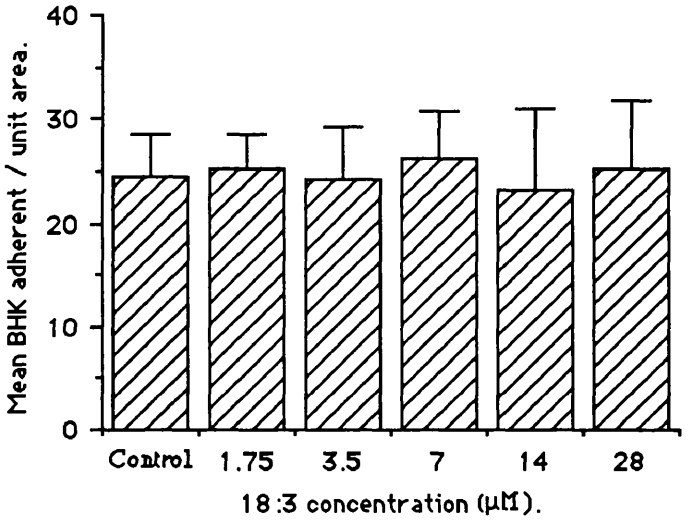


Fig.3.17. The dose response effect of linolenoyl-CoA (18:3) on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with linolenoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 20 standard measurements. Error bars = one standard deviation.

Table.3. Linolenoyl-CoA effect on BHK cell adhesion to polystyrene TC dishes in serum-free medium in presence of EDTA.

Linolenoyl-CoA (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
1.75	103 ± 16.56
3.50	99 ± 20.57
7.00	107 ± 18.56
14.0	94 ± 32.35
28.0	104 ± 26.83

BHK cells at concentration of 0.2×10^6/ml were first preincubated with 3.5 μM linolenoyl-CoA, 1.25×10^{-5} M ATP and 2.5 mM EDTA for a period of 20 mins at 37°C in serum-free Ham's F-10. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

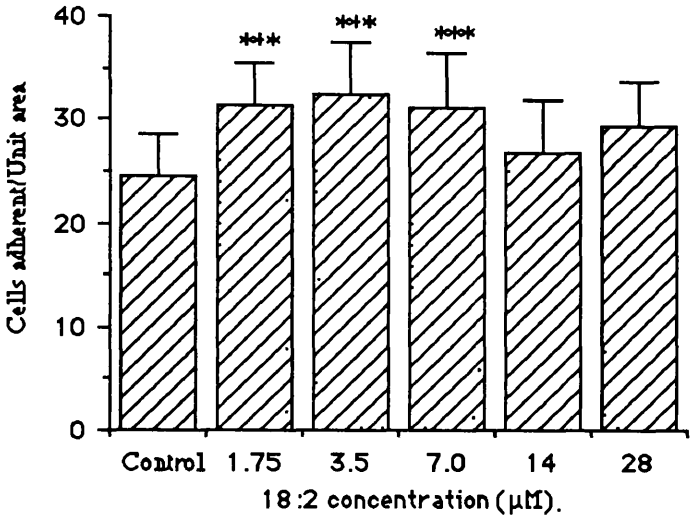


Fig.3.18. The Dose response effect of linoleoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with linoleoyl-CoA and $1.25x10^{-5}$ M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 20 standard measurements. Error bars are one standard deviation.

*** Differ significantly from the control *(t-test), P< 0.001.*

Table.4. Linoleoyl-CoA (18:2) effect on BHK cell adhesion to polystyrene TC dishes in serum-free medium in presence of EDTA.

18:2 (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
1.75	150 ± 29.89 (***)
3.50	139 ± 27.64 (***)
7.00	144 ± 22.15 (***)
14.0	96 ± 19.33
28.0	103 ± 17.18

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM linoleoyl-CoA, $1.25x10^{-5}$ M ATP and 2.5 mM EDTA for a period of 20 mins at 37°C in serum-free Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were removed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and the adherent cells were then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.5. Linolenoyl-CoA effect on BHK cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

Linolenoyl-CoA (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
3.5	98 ± 20.06
7.0	92 ± 12.04
14	92 ± 13.43
28	96 ± 13.50

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM stearoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in 3% serum Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After that, unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

Table.6. Linoleoyl-CoA (18:2) effect on BHK cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10.

concentration (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
3.50	102 ± 19.25
7.00	94 ± 13.28
14.0	92 ± 13.43
28.0	96 ± 13.52

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM linoleoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in 3% serum Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

Then, the cells were plated into polystyrene TC dishes incubated for 20 mins at 37°C. The number of adherent cells was then counted. BHK cells did not show any alteration in their adhesion at concentrations lower than 7μM. Nevertheless, higher concentrations than 7μM decreased BHK cell adhesion significantly from control samples (Fig.3.19).

In a similar experiment BHK cells were pretreated in serum-free conditions with 2.5 mM EDTA in mixture with oleoyl-CoA and ATP for 20 mins at 37°C, then the cells were added to polystyrene TC dishes, incubated for 20 mins at 37°C, and the adherent cells were counted. Concentrations lower than 7μM enhanced BHK cell adhesion, while concentrations higher than that, decreased the adhesion significantly ($P<0.001$)(Table. 7).

ii. Cell spreading.

Oleoyl-CoA had a marked effect on BHK spreading area when it is added in combination with 12.5 μM ATP in serum-free medium (Table.8) and (plate.4).

iii. Adhesion in presence of serum.

The adhesion and the spreading area of BHK cells was not affected by oleoyl-CoA in 3% serum Ham's F-10 conditions (Table. 9). It should be noted that stearoyl-CoA and oleoyl-CoA used at high concentrations reduced BHK cell viability (Table. 10).

3.1.3. The effect of free fatty acids on BHK cell adhesion.

Both Curtis et al., 1975a,b,c, and Hoover et al., 1977, had found marked changes in adhesion of other cell types treated with free fatty acids which were applied in solution with CoA and ATP, and had also demonstrated incorporation of these fatty acids into

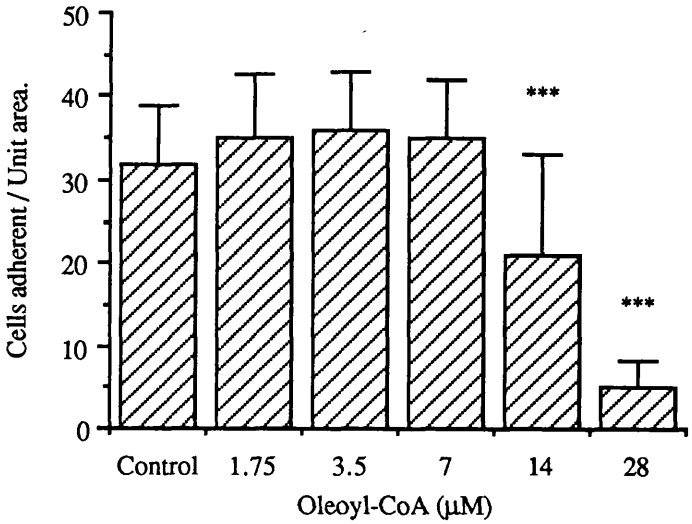


Fig.3.19. The dose response effect of oleoyl-CoA on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with oleoyl-CoA and 1.25×10^{-5} M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 40 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), P< 0.001.*

Table.7. Oleoyl-CoA (18:1) effect on BHK cell adhesion to polystyrene TC dishes in serum-free Ham's F-10.

Oleoyl-CoA (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
1.75	144 ± 29.33 (***)
3.50	111 ± 22.76
7.00	108 ± 18.73
14.0	74 ± 21.46 (***)
28.0	5 ± 3.60 (***)

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM oleoyl-CoA, $1.25x10^{-5}$ M ATP and 2.5 mM EDTA for a period of 20 mins at 37°C in 3% serum Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

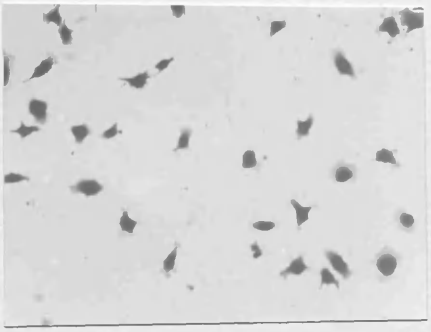
Table.8. Inhibition of BHK fibroblast cell spreading on polystyrene TC dishes in serum-free Ham's F10 medium over 20 mins incubation at 37°C with oleoyl-CoA.

Oleoyl-CoA (μM).	Mean spreading area/per cell (μm^2) ± Standard deviation
Control	430 ± 212.68
ATP	566 ± 302.81
1.75 + ATP	315 ± 195.34 ***
3.50 + ATP	281 ± 194.27 ***
7.00 + ATP	221 ± 193.72 ***
14.00 + ATP	151 ± 90.94 ***

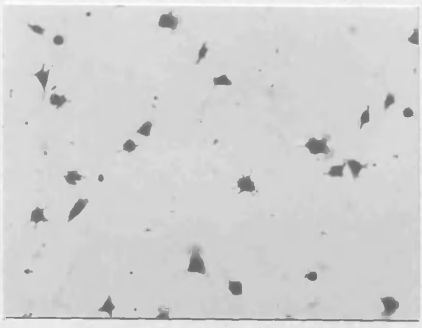
BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with oleoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in serum-free Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for (60-65) mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and the area of each adherent cell was measured. Cells were visualized with x10 objective and spreading is expressed as mean area per cell (μm^2) ± standard deviation. More than 500 cells were measured for each experimental condition in three replicate TC dishes.

***Differ significantly from the control *(t-test), P<0.001.*

Plate.4. The dose response effect of oleoyl-CoA on BHK cell spreading.
Cells were preincubated for 20 mins at 37°C, in serum-free medium (control) (a), or with ATP and [1.75 μM (b), 3.5 μM (c), 7 μM (d) or 14 μM oleoyl-CoA (e)]. Cells were then plated into polystyrene tissue culture dishes and incubated for 65 mins at 37°C. After this period, cells were fixed with formal saline, stained with kenacid blue and then photographed.
Scale bar = 100 μm.

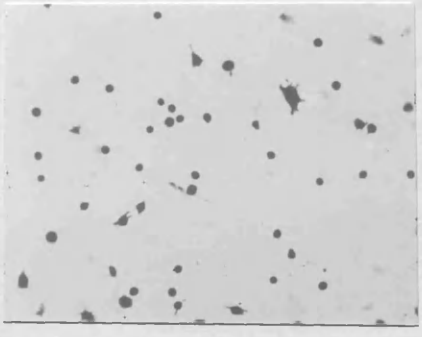


a

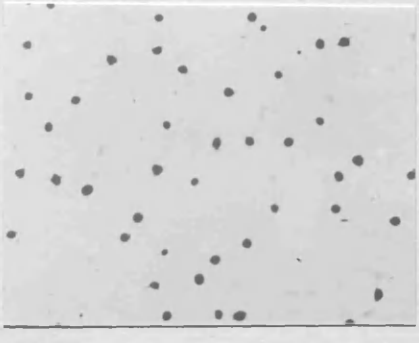


b

c



d



e

Table.9. Oleoyl-CoA (18:1) effect on BHK cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10.

Oleoyl-CoA (μM).	Cells adherent (% of the control) ± Standard deviation
Control	100
3.50	92 ± 11.50
7.00	99 ± 14.73
14.0	95 ± 18.97
28.0	97 ± 14.95

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with 3.5 μM oleoyl-CoA and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C in 3% serum Ham's F-10. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as percentage of control samples. Means of 20 standard measurements ± standard deviation.

Table.10. Stearoyl-CoA and oleoyl-CoA (18:1) effect on BHK cell viability in serum-free Ham's F-10.

Acyl-CoA concentration (μM)	Viability (%)
Stearoyl-CoA	
0	94
14	25
28	22
Oleoyl-CoA	
0	94
14	89
28	48

BHK cells were first preincubated in 20 ml glass universals with 14 and 28 μM oleoyl-CoA or stearoyl-CoA and 1.25 x 10^{-5} M ATP for a period of 20 mins at 37°C in serum-free Ham's F-10. After this period the cells were resuspended in 1 ml serum-free Ham's F-10 and the viability was tested using trypan blue exclusion method.

phosphatidyl lipids unlike my results with the acyl-CoA compound. In consequence my experiments were repeated with the free acids. Linoleic and arachidonic acid were used as the free acid form and dissolved in a minimum concentration of ethanol at final concentration less than 1 %.

3.1.3-1. Linoleic acid.

The first experiment was carried out in serum-free conditions. 12.5 μM ATP, 5 μM CoA and linoleic acid (μg/ml) were added to the cells as described before, preincubated for 20 minutes at 37°C in glass universals, then the adhesion was measured after 20 minutes incubation in polystyrene TC dishes. The results (fig.3.20) show clearly a marked decrease in the adhesion at both concentrations 10 and 20 μg/ml.

In a similar experiment in which 3 % serum Hams F-10 was used to stop tryptic activity, BHK cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 was also significantly decreased (Fig.3.21).

3.1.3-2. Arachidonic acid.

The effect of arachidonic acid, the precursor of prostaglandins and leukotrienes, on BHK cell adhesion was also examined and compared with the effect of its analogue arachidonoyl-CoA on BHK cells as well.

Arachidonic acid in combination with ATP and CoA, reduced cell attachment in a dose dependent manner in both serum-free and serum-containing medium (Fig.3.22 & 3.23). Viability test however, revealed that both linoleic and arachidonic acid used at 10 μg/ml and higher reduced the viability to approximately 5%, especially in serum-free medium. The effect was less pronounced when serum was used instead of serum-free medium. In fact 10μg/ml of

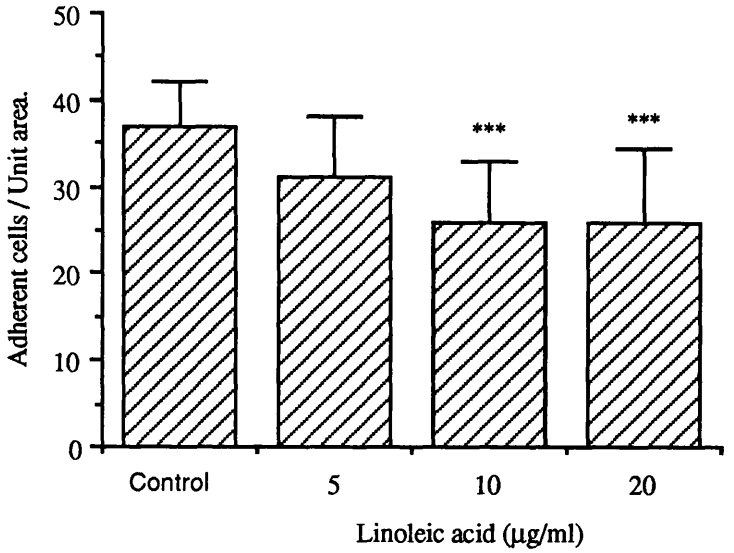


Fig.3.20. The effect of linoleic acid on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with linoleic acid, $5x10^{-6}$ M Coenzyme A and $1.25x10^{-5}$ M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 20 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), P< 0.001.*

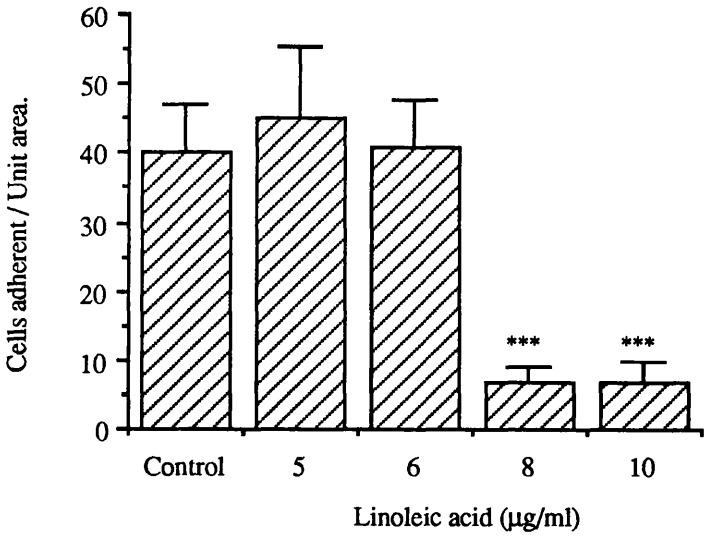


Fig.3.21. The effect of linoleic acid on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml glass universals with linoleic acid, 5×10^{-6} M Coenzyme A and 1.25×10^{-5} M ATP in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 2.5×10^{-3} cm^2. Means of 20 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), $P < 0.001$.*

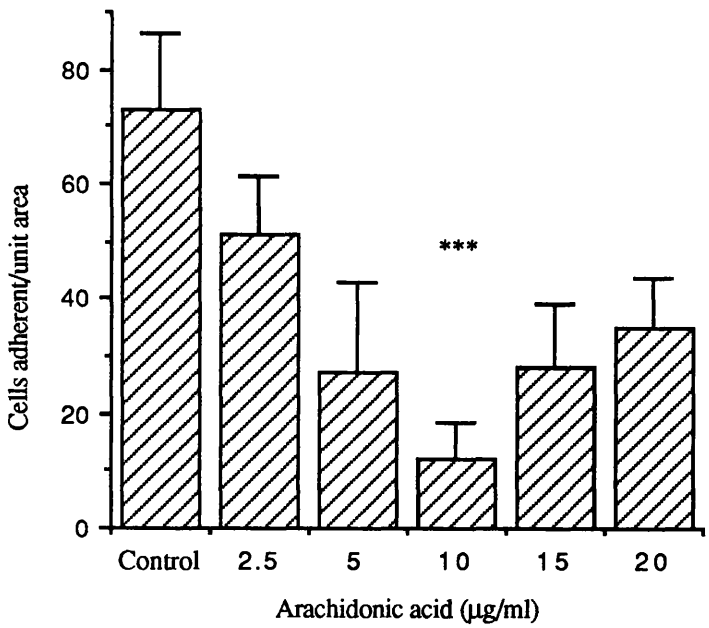


Fig.3.22. The effect of arachidonic acid on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml glass universals with arachidonic acid, $5x10^{-6}$ M Coenzyme A and $1.25x10^{-5}$ M ATP in serum-free Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Mean of 60 standard measurements for each experimental condition. Error bars = one standard deviation.

*** All bars differ significantly from the control, *P<0.001, (t-test)*.

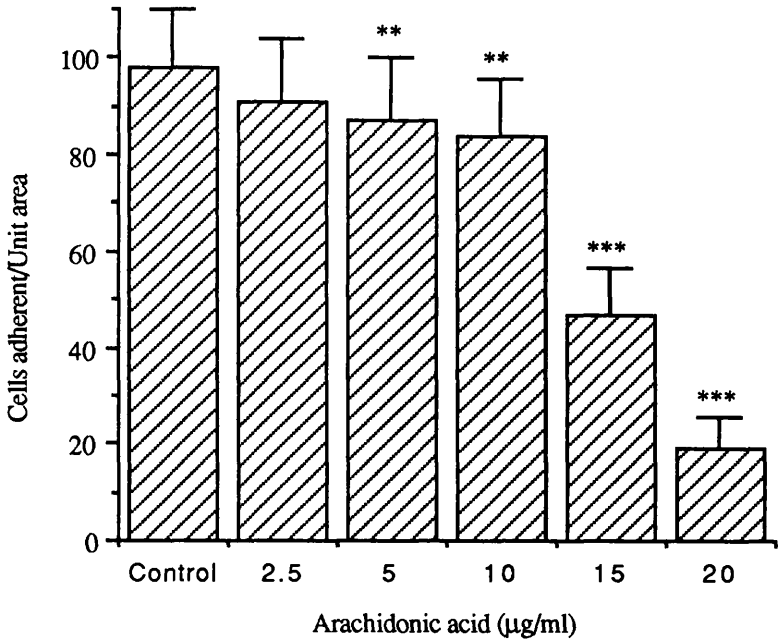


Fig.3.23. The effect of arachidonic acid on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

Cells at concentration of $0.2x10^6$/ml were preincubated in glass universals with different concentrations of arachidonic acid, $5x10^{-6}$ M Coenzyme A and $1.25x10^{-5}$ M ATP for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Mean of 30 standard measurements. Error bars = one standard deviation.

; * Differ significantly from the control, *(t-test), $P<0.01$ & 0.001, respectively.*

arachidonic acid did not affect the viability at all in serum-containing medium.

It is of great importance to establish whether arachidonic acid affect BHK cell adhesion under flow the same manner as it did under static conditions. Accordingly, arachidonic acid was tested for its ability to alter the adhesiveness of BHK cells under flow conditions.

There was an overall decrease in BHK cell adhesion under flow conditions when arachidonic acid was used at 10 and 20 μg/ml (Fig. 3.24).

The above results show that the effect of fatty acids used in the free acid form is different from the effect of fatty acyl-CoA. At the time where no effect was seen with linoleoyl-CoA and arachidonoyl-CoA, both linoleic and arachidonic acid generally decreased cell adhesion in significant and clear manner, but viability was suspect.

It can be concluded from this section that among the fatty acyl-CoAs used, stearoyl-CoA and oleoyl-CoA are the two main fatty acyl-CoAs that had a clear effect on cell adhesion as well as on the spreading at approximately the same concentrations in serum-free conditions. The other acyl-CoAs in general kept the adhesion at control level (see comparison summary in Fig.3.25). The inability of some of these acyl-CoAs to alter the adhesion in serum-free conditions, suggests that the incorporated acyl-CoAs might have been exchanged in the plasmalemma with the same fatty acids released or they could have been used for metabolic purposes (see discussion).

It should be mentioned however, that all the acyl-CoAs used were unable to modify either the adhesion or the spreading in

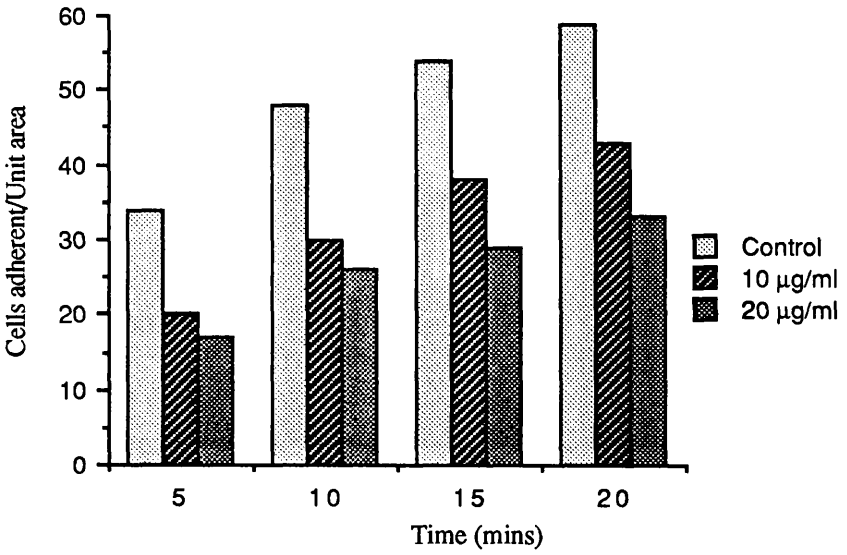


Fig.3.24. The effect of arachidonic acid on BHK fibroblast cell adhesion to glass in 3% serum Ham's F-10 medium under flow conditions.

BHK cells at concentration of $2x10^6$/ml were first preincubated in 20 ml glass universals with 10 or 20µg/ml arachidonic acid, $5x10^{-6}$ M coenzyme A and $1.2x\ 10^{-5}$ M ATP in 3% serum Ham's F-10 for 20 mins at 37°C. The suspension of BHK cells were then perfused through the chamber with a mechanical syringe driver at known flow rate (1.02 ml/hour), placed on the stage of an inverted Leitz microscope, under 37°C. Attached cells were counted at different times as described in Methods. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Values are the mean of two replicates.

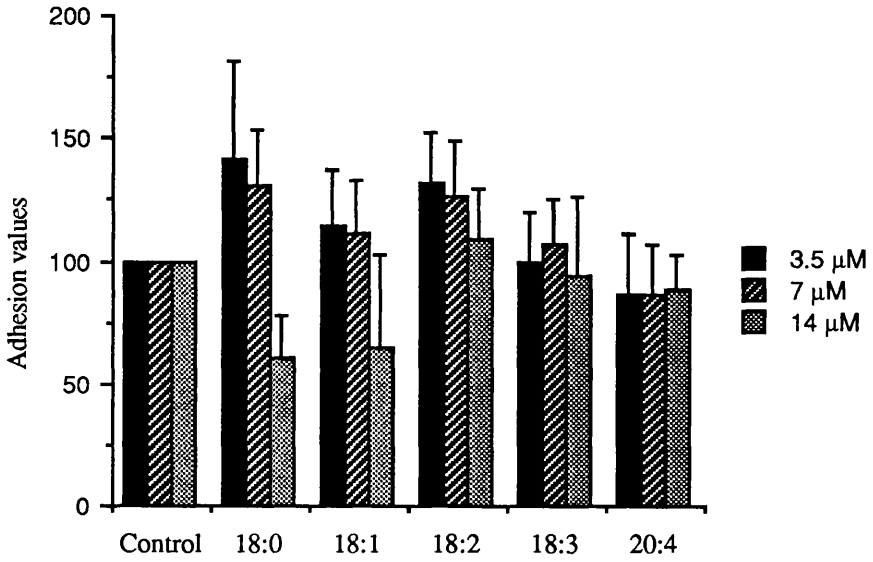


Fig.3.25. A comparison summary of the effect of various acyl-CoA on the attachment of BHK cells to polystyrene TC dishes in serum-free medium.

Adhesion values are expressed as percentage of the control. 18:0, Stearoyl-CoA; 18:1, Oleoyl-CoA; 18:2, Linolenoyl-CoA; 18:3, Linolenoyl-CoA; 20:4, Arachidonoyl-CoA.

serum-containing media. This may suggest the presence of either an acyl binding agent in the serum or the presence of an inhibitor or lipids are present in the medium which prevented the access of stearoyl-CoA to the membrane. This matter was investigated by incorporation experiments (see later).

3.1.4. The incorporation of oleoyl-CoA into BHK cell plasma membrane.

^{14}C-labeled oleoyl-CoA was used to test whether BHK cells incorporated the acyl-CoAs into their plasmalemmal lipids after the 20 mins incubation.

The total oleoyl-CoA incorporated into the cells and their plasma membranes was measured as described in Methods.

3.1.4-1. Incorporation in serum-free conditions.

In this experiment leupeptin rather than serum was used to stop trypsin activity and the incorporation study was carried out in serum-free conditions.

BHK cells were incubated for 20 mins at 37°C in serum-free medium with $5x10^{-6}$ M ^{14}C-oleoyl-CoA (specific activity, 52 mCi/mmol), washed, and then the total oleoyl-CoA incorporated into cells was measured by taking an aliquot and measuring the radioactivity in it. The plasma membrane was then isolated and the total oleoyl-CoA incorporated into it was also counted.

The results shown in Table (11) reveal that approximately 40-50 % of the total label is incorporated into the whole cells in each individual experiment. 21% of the total incorporated is present in the plasma membrane. This represent approximately 8% of the total used.

Extraction and identification of plasmalemmal lipids were also made (see Methods). Lipid separation, using thin layer

Table.11. The uptake of (^{14}C)-oleoyl-CoA by intact BHK fibroblasts in serum-free medium.

	dpm	% of the total
Total used	6812953	
Free	1671505	26%
Total incorporated	2735000	40%
Total in plasmalemmal	552960	8%

BHK cells at concentration of $35x10^6$/ml were incubated in glass universal in 10 ml serum-free incubation medium which consists of 5 μM (^{14}C)-oleoyl-CoA (specific activity, 52.2 mci/mmol) and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C. After this period, BHK cells were washed first with cold oleoyl-CoA and then with (0.15 M NaCl, 30 mM $NaHCO_3$ at pH.7) and resuspended in $NaHCO_3$ pH.7. Total radioactivity in cells as well as in plasma membrane were then counted. Results are expressed as dpm per 35 $x10^7$ cells, a representation of one of two assays.

chromatography (plate.5), show that the incorporation is into lysophospholipids (LP), phospatidylcholine (PC), phosphatidyl-ethanolamine (PEA), phosphatidylinositol (PI), phosphatidylserine (PS), neutral lipids (NL) and other unidentified components (Table.12).

3.1.4-2. Incorporation in serum-containing medium.

This experiment was carried out in 3% serum Ham's F-10 instead of serum-free condition to test whether serum is affecting the incorporation of the acyl-CoAs.

Similarly, BHK cells incubated for 20 mins with oleoyl-CoA were found to incorporate oleoyl-CoA into their plasma membrane in 3% serum Ham's F-10. Results presented in Table (13) shows that approximately 23% of the total oleoyl-CoA was taken up by the cells over the 20 mins incubation. 3% of the total oleoyl-CoA was incorporated into plasmalemma.

It is clear from the incorporation experiments that intact BHK cells incorporated a substantial quantity of the acyl-CoA to its plasma lemma after the 20 mins incubation in serum-free as well as in serum containing medium. This might suggest that the observed effect on cell adhesion and spreading is due to an alteration in the plasma membrane properties. It should be noted however, that the uptake of oleoyl-CoA in serum-containing medium was reduced by approximately 43% in comparison with the incorporation in serum-free medium. This difference may explain the lack of effect of acyl-CoAs in serum-rich medium.

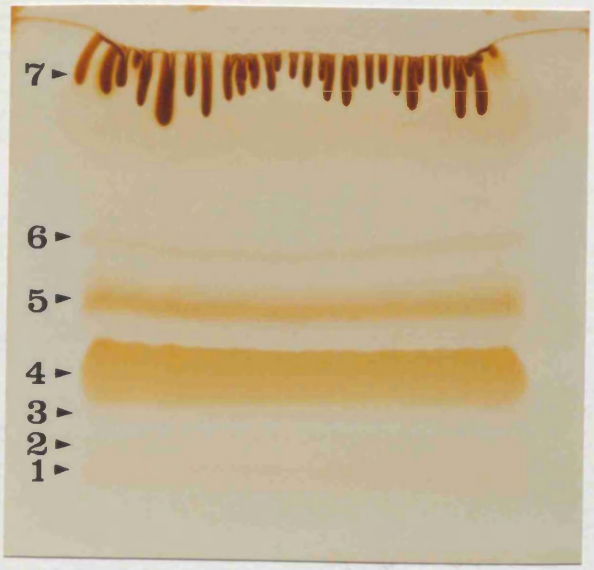


Plate.5. Separation and identification of lipids using thin layer chromatography (TLC).
Lipids extracted from plasma membrane with methanol/ chloroform were separated on TLC plates using chloroform/methanol/ acetic acid/ water as solvent system. Lipid separated in this way were detected non-destructively with iodine vapour.

1: origin; 2: lysophospholipids; 3: phosphatidylinositol; 4: phosphatidylcholine;5:phosphatidylserine + others; 6: phosphatidyl-ethanolamine; 7: solvent front (oleic acid + others).

Table.13. The uptake of (^{14}C)-oleoyl-CoA by intact BHK fibroblasts in 3% serum-containing medium.

	dpm	% of the total
Total used	5651672	
Free	2969863	53%
Total incorporated	1301234	23%
Total in plasmalemmal	191667	3%

BHK cells at concentration of $35x10^6$/ml were incubated in glass universal with 10 ml incubation medium which consists of 5 μM (^{14}C)-oleoyl-CoA (specific activity, 52.2 mci/mmol) and $1.25x10^{-5}$ M ATP for a period of 20 mins at 37°C. The cells were after that washed first with cold oleoyl-CoA and then with (0.15 M NaCl, 30 mM $NaHCO_3$ at pH.7) and resuspended in $NaHCO_3$ pH.7. Total radioactivity in cells as well as in plasma membrane were then counted. Results are expressed as dpm per 35 $x10^7$ cells.

3.2. Cell activation and adhesion.

Activation of cell functions, in particular cell adhesion, is a very well established phenomenon for myeloid cells as well as other cell types. Leukocytes, for instance can be activated to adhere to different substrata after being non-adherent. Factors responsible for this activation include diacylglycerol, inositol trisphosphate and arachidonic acid metabolites. The phorbol esters and the calcium ionophores are also a very well known activators of cell adhesion of many cell types.

Therefore it was of great relevance to establish whether fibroblastic cells can have their adhesion activated in a similar manner using the above factors.

3.2.1. The effect of arachidonic acid metabolites and PLA_2 inhibitors on BHK cell adhesion.

3.2.1-1. The effect of bromophenacyl bromide and mepacrine on BHK cell adhesion

i. Bromophenacyl Bromide (BPB) a potent inhibitor of phospholipase A_2 in cell-free systems (Roberts et al., 1977) was the first to be tested. In this assay, BHK cells were first preincubated for 20 mins at 37°C with different concentrations of BPB (2-8μM), and then, plated into polystyrene TC dishes, incubated for 20 mins at 37°C and the adherent cells were counted.

The attachment of BHK cells treated with BPB was reduced sharply in serum (Fig.4.1; plate. 6) and serum-free (Fig.4.2) conditions. BHK cells treated with BPB then washed and resuspended in fresh medium, also did not adhere to polystyrene TC dishes even after a long period of incubation. There was an irreversible inhibition of cell adhesion.

I sought to investigate the effect of BPB on already attached

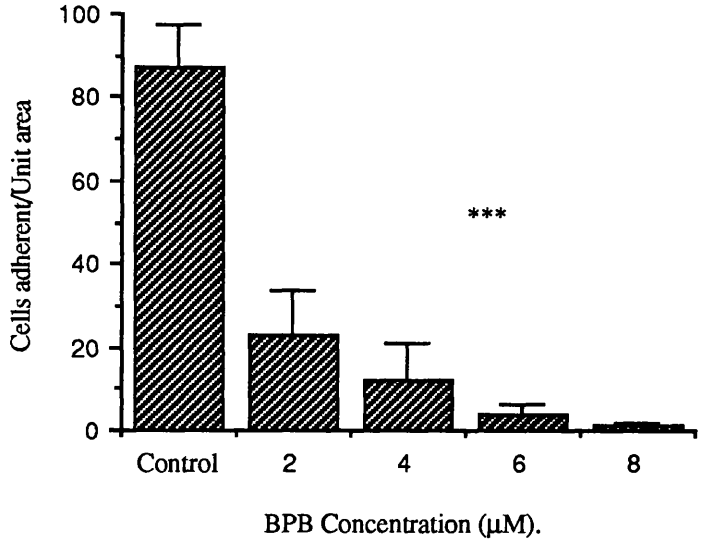


Fig.4.1. The effect of bromophenacyl bromide (BPB) on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml bromophenacyl bromide in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** All bars differ significantly from the control *(t-test), P< 0.001*.

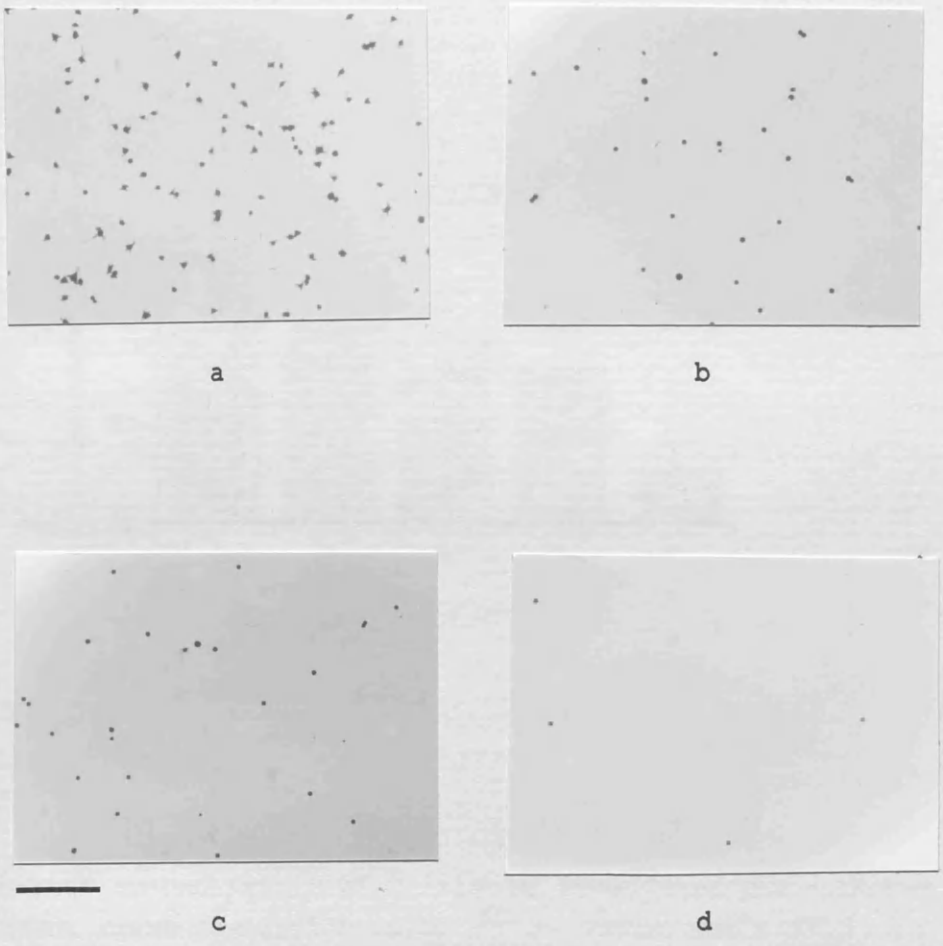


Plate. 6. The effect of bromophenacyl bromide (BPB) on BHK cell adhesion in 3% serum-containing medium.
Cells were preincubated in 3% serum Ham´s F-10 without (control) (a), or with 2 μM BPB (b) or 4 μM (c) for 20 mins at 37°C, then plated into polystyrene tissue culture dishes and incubated for 20 mins at 37°C. After that, adherent cells were fixed, stained, and photographed.

Scale bar = 200 μM

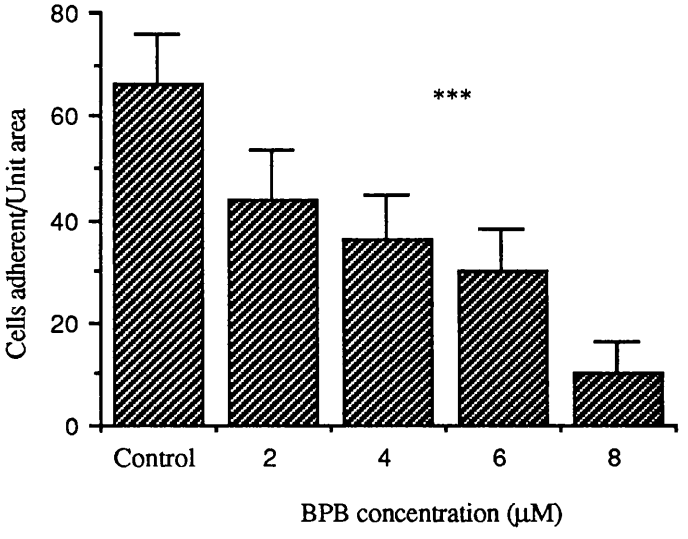


Fig.4.2. The effect of bromophenacyl bromide (BPB) on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

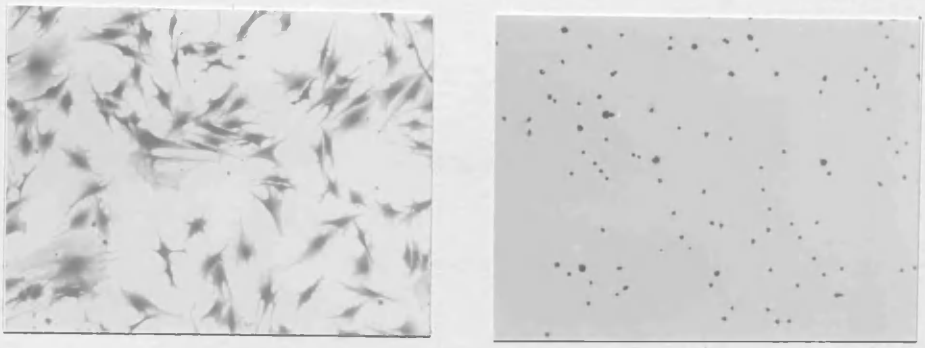
BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml with Bromo-phenacyl-bromide in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** All bars differ significantly from the control *(t-test), $P< 0.001$.*

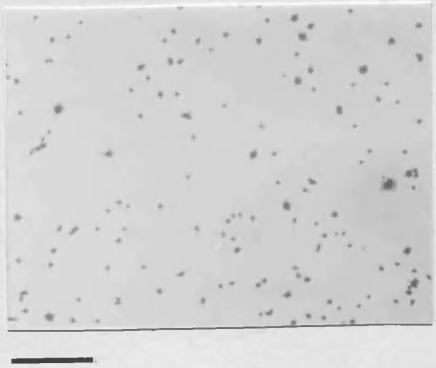
cells. In this experiment, untreated BHK cells were incubated for a period of 75 mins at 37°C in 3% serum Ham's F-10 and then different concentrations (2,4, μM) of BPB were added. The mixture was incubated for up to 24 hours. The cells were checked at different intervals and the differences were recorded. One of the observations, is that though adherent cells treated with any concentration of BPB did not detach, the spreading was entirely inhibited and the cells did not grow any further in comparison with control samples (plate. 7). The viability test, using trypan blue exclusion method, revealed that up to 8 μM BPB did not affect cell viability (Table. 14).

ii. The effect of Mepacrine, another phospholipase A_2 inhibitor (McCrea et al., 1985), on BHK cell attachment was also examined.

BHK cell adhesion was reduced by approximately 20% of control values when cells were preincubated for 20 mins at 37°C with mepacrine in 3% serum Ham's F-10 conditions prior to the attachment assay (Fig.4.3). A similar results were seen when the experiment is performed in serum-free Ham's F-10 (Fig.4.4). The effect of BPB and mepacrine on cell adhesion could be due to the inhibition of PLA_2 activity. The inability of mepacrine however, to reduce the adhesion to a level similar to that observed with BPB, may reflect a partial inhibition of PLA_2 unlike bromophenacyl bromide. It should be noted that there is no direct evidence that BPB and mepacrine inhibited PLA_2 activity in the above experiments. However, the assumption that the effect on cell adhesion could be due to the inhibition of PLA_2 activity, is based on the vast literature showing the ability of these compounds to inhibit phospholipase A_2. Therefore, this mater is open for further investigation.



a b



c

Plate. 7. The effect of bromophenacyl bromide (BPB) on already attached BHK cells.

Cells were first added to polystyrene tissue culture dishes, incubated for a period of 75 mins at 37°C and then BPB was added. Cells were removed after 24 hours, fixed, stained and photographed.

a. Control; b. 2μM BPB; c. 4μM BPB.

Scale bar = 200 μM

Table.14. The effect of bromophenacyl bromide on BHK cell viability over the 20 mins incubation.

Bromophenacyl bromide (μM)	Viability (%)
0.0	96
2.0	93
4.0	95
6.0	93
8.0	94

BHK cells were incubated in 20 ml glass universals with bromophenacyl bromide for 20 mins at 37°C in 3% serum Ham's F-10. Then, cells were centrifuged and resuspended in 1 ml fresh 3% serum Ham's F-10 and the viability was measured using trypan blue exclusion method.

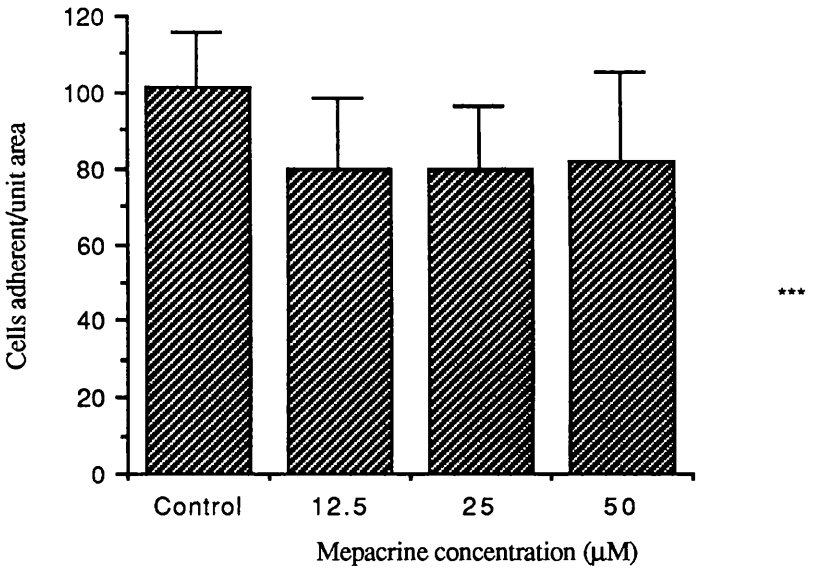


Fig.4.3. The effect of mepacrine on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

BHK cells at concentration of $0.2x10^6$/ml were first preincubated in 20 ml siliconized glass universals with mepacrine in 3% serum Ham's F-10 for 20 mins at 37°C. Then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** All bars differ significantly from the control *(t-test), P< 0.001.*

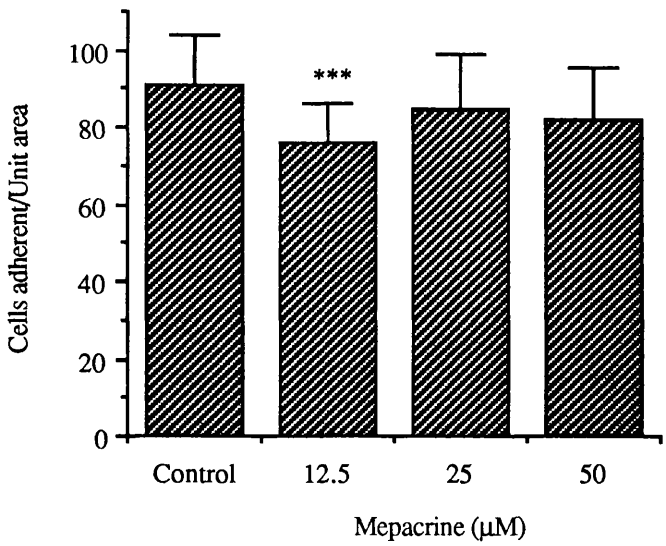


Fig.4.4. The effect of mepacrine on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F-10 medium.

BHK cells at concentration of 0.2×10^6/ml were first preincubated in 20 ml siliconized glass universals with mepacrine in serum-free medium for 20 mins at 37°C. Then, 3 ml (0.2×10^6/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 6.3×10^{-3} cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

*** Differ significantly from the control *(t-test), $P < 0.001$.*

3.2.1-2. The effect of arachidonic acid metabolites on BHK cell adhesion.

Eicosanoids are derived mostly from fatty acids containing 20 carbon atoms notably arachidonic acid. They include prostaglandins thromboxanes and leukotrienes. These substances are produced locally when required and usually rapidly destroyed. They are involved in the regulation of a large number of functions including intracellular calcium levels, chemotaxis, relaxation of smooth muscles and cell adhesion.

Because of the vast range of effects of this substances on many cell functions including cell adhesion, it was interesting to test their effect on the adhesion of BHK fibroblasts.

i. Prostacyclin (PGI_2).

Prostacyclin (PGI_2), an arachidonic acid metabolite, known to have an inhibitory effect on platelet aggregation and PMN leukocyte adhesion (see earlier). In order to establish whether BHK cells will respond in similar manner, their adhesion to polystyrene TC dishes in the presence of prostacyclin was examined.

In this experiment leupeptin was used to stop tryptic activity, and cells were resuspended in fresh serum-free Ham's F-10 and set at $0.2x10^6$/ml. Cells were preincubated for 10 mins at 37°C in glass universals with different concentrations of PGI_2, then, 3 ml of cells were added to each of three replicate dishes, incubated for 20 mins at 37°C and the adherent cells were counted. The results shown in (Fig. 4.5) show clearly that BHK cell adhesion was not altered.

The effect of PGI_2 and prostaglandin E_2 (PGE_2) on BHK cell adhesion was investigated in 3% serum Ham's F-10.

Both, PGI_2 and PGE_2 did not affect BHK cell adhesion in 3% serum Ham's F-10 (Fig.4.6a). However, the spreading area per cell was increased (Fig.4.6b). The increase in cell spreading was more

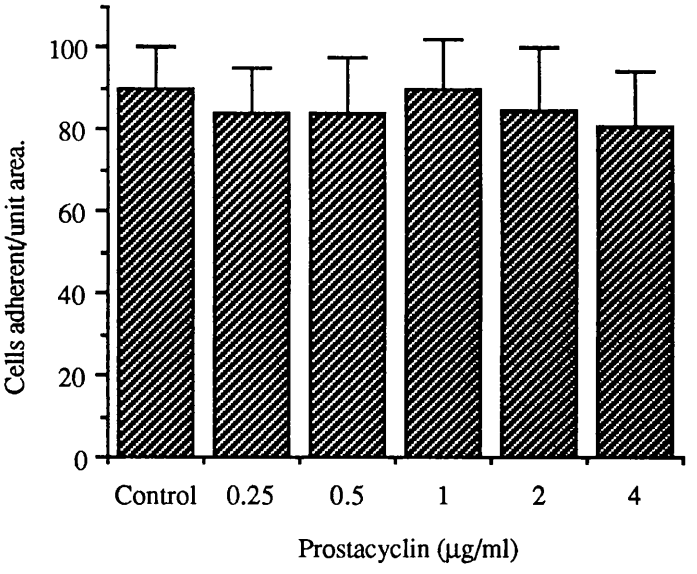
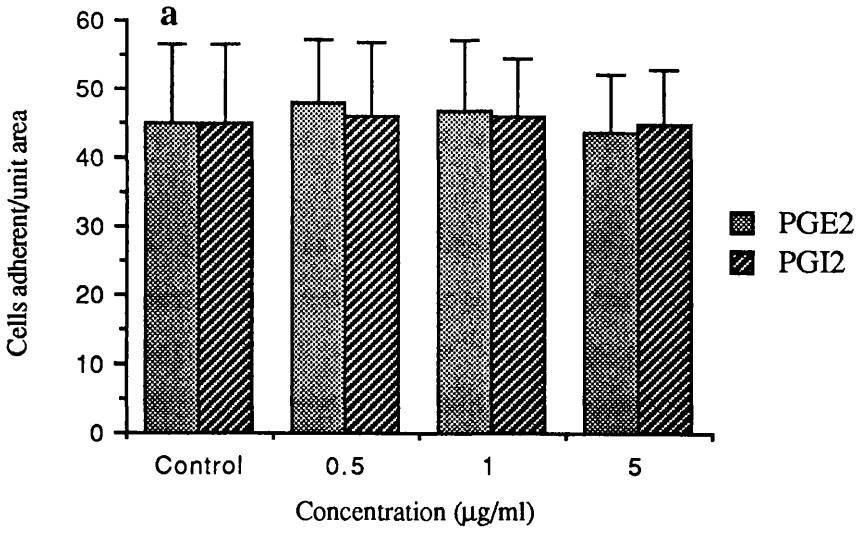


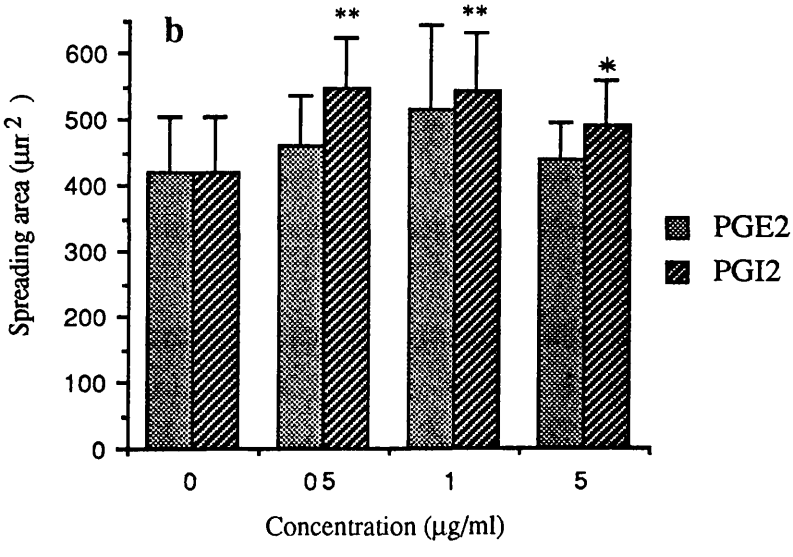
Fig.4.5. The effect of prostacyclin on BHK cell adhesion to polystyrene dishes in serum-free Ham's F-10.

BHK cells were trypsinised and leupeptin used to neutralize trypsin activity, spun for 5 mins, resuspended in fresh serum-free Ham's F-10 medium and set at $0.2x10^6$/ml in 20 ml glass universals. PGI_2 was then added to the cells and preincubated for 10 mins at 37°C. 3 ml ($0.2x10^6$/ml) cells were after that added to each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $6.3x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

Fig.4.6. The effect of prostacyclin and prostaglandin E_2 on BHK cell adhesion (a) and cell spreading (b) in 3% serum Ham´s F-10 medium.

BHk cells were first incubated with PGI_2 or PGE_2 for 10 mins at 37°C. 3 ml ($0.2x10^6$/ml) cells were afterward added to each polystyrene TC dish, and incubated for 20 mins at 37°C. Unattached cells after that were gently washed out with Hepes saline, and adherent cells were fixed, stained, counted and their spreading area measured. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 20 standard measurements.
The spreading area is expressed as the mean area per cell (μm^2). More than 400 cells were counted for each experimental condition.
Error bars = one standard deviation.
*, ** Differ significantly from the control (*t-test*), *P< (0.05 and 0.01 respectively).*


a
Cells adherent/unit area
60
50
40
30
20
10
0
Control
0.5
1
5
Concentration (µg/ml)
PGE2
PGI2


b
Spreading area (µm 2)
600
500
400
300
200
100
0
**
**
*
0
05
1
5
Concentration (µg/ml)
PGE2
PGI2

pronounced with PGI_2 than PGE_2. The lack of effect of these prostaglandins on BHK cell adhesion is unclear. It could be argued however, that their effect might have been quickly regulated. Therefore, cell adhesion was kept at control level.

ii. Leukotriene B_4. It is an another arachidonic acid metabolite, and a PMN leukocyte activator, known also for its ability to induce calcium mobilization and increase the interaction between PMN and endothelia. It was tested on BHK and endothelial (B10.D2) cell aggregation.

In this experiment, flasks containing 4 ml cells ($1x10^6$/ml) were incubated on a shaker at 37°C with LTB_4. Samples were removed at different times, starting from time zero and number of cells single or in aggregates were counted (fig.4.7; 4.8).

In these two figures, the time of incubation is plotted versus number of cells at any time divided by number of cells at the starting time. These results showed that LTB_4 at concentration 1.25 and 0.125μM enhanced BHK and B10.D2 cell-cell adhesion in comparison with untreated cells. The effect was however more pronounced on BHK cells than endothelial cells. The mechanism involved may be related to the ability of LTB_4 to increase intracellular calcium which might have led in a direct or indirect manner to the stimulation of cell-cell adhesion.

3.2.2. Calcium/protein kinase C pathway and BHK cell adhesion.

Calcium mobilization and protein kinase C activation usually act synergistically to activate cell functions such as superoxide anion generation, enzyme secretion, phagocytosis, cytotoxicity and adhesion. Many agents are known to mobilise calcium from intracellular stores or from extracellular milieu, and other substances were shown to activate in a direct or indirect manner

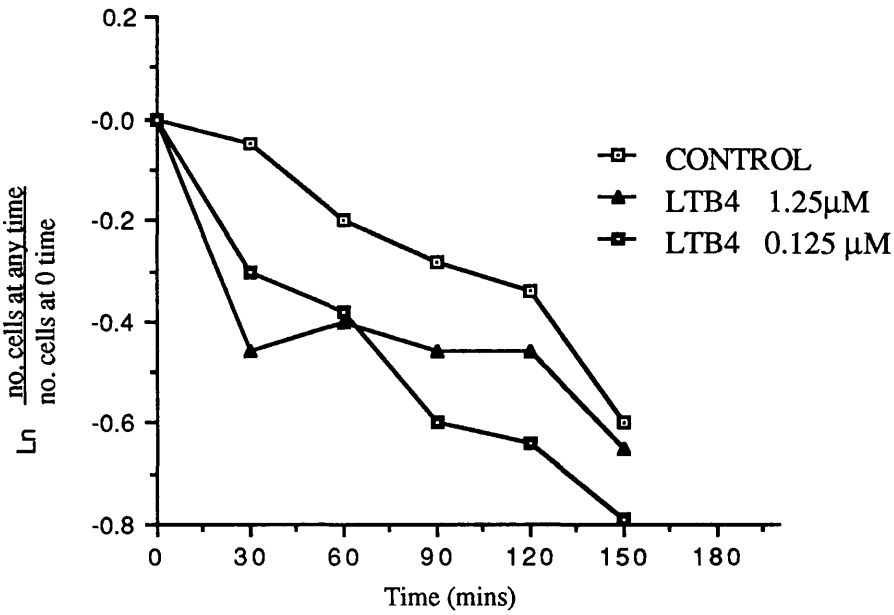


Fig.4.7. Time course curve of LTB_4 effect on BHK cell aggregation in 3% serum Ham's F-10 medium.

BHK cells were first trypsinised and 5 ml 3% serum Ham's F-10 used to neutralize trypsin activity, span at 1500 rpm for 5 mins, resuspended in fresh 3% serum Ham's F-10 medium and set at $1x10^6$/ml. LTB_4 was then added to the cells at final concentration of 1.25 μM and 0.125 μM. 4 ml ($1x10^6$/ml) cells were added to each 10 ml conical glass flask and incubated on a shaker at 37°C to allow cell-cell contact. Samples were removed at different times and the number of cells (single or in aggregates) was counted as described in methods. Each point is the mean of 40 standard measurements. Standard deviations are approximately 20% of the mean values.

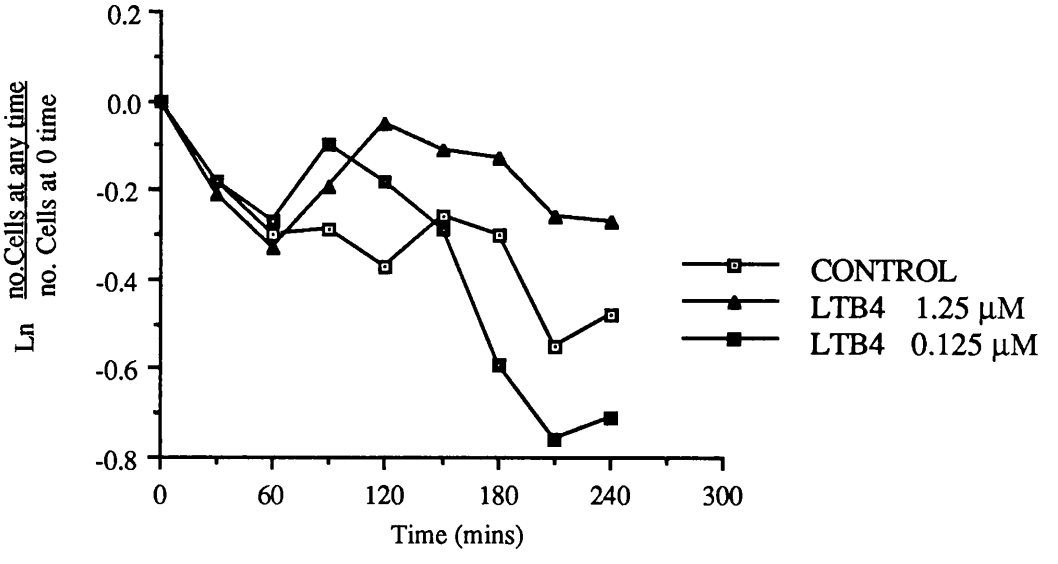


Fig.4.8. Time course curve of LTB_4 effect on B10.D2 endothelial cell aggregation in 3% serum Ham's F-10 medium.

B10.D2 endothelial cells were first trypsinised and 5 ml 3% serum Ham's F-10 used to stop trypsin activity, span at 1500 rpm for 5 mins, resuspended in fresh 3% serum Ham's F-10 medium and set at $1x10^6$/ml. LTB_4 was then added to the cells at final concentration of 1.25 µM and 0.125 µM. 4 ml ($1x10^6$/ml) cells were added to each 10 ml conical glass flask and incubated on a shaker at 37°C to allow cell-cell contact. Samples were removed at different times and the number of cells (single or in aggregates) was counted as described in methods. Each point is the mean of 20 standard measurements. Standard deviations did not exceed 20% of the mean values.

protein kinase C. Among these, are inositol trisphosphate and diacylglycerol, phorbol esters and calcium ionophores.

3.2.2-1. The effect of diacylglycerol kinase inhibitor (R59022) on cell adhesion.

Diacylglycerol is converted rapidly to phosphatidic acid by diacylglycerol kinase (ATP:1,2diacylglycerol 3-phospho-transferase) (Lapetina & Cuatrecasas 1979). Because diacylglycerol kinase converts diacylglycerol to phosphatidic acid, it may play an important role in regulating the coupling of agonist-induced generation of membrane diacylglycerol to activation of protein kinase C (Besterman et al., 1986).

A diacylglycerol kinase inhibitor (6-[2-[4-[(4-fluorophenyl) phenylmethelene]- 1-piperidinyl] -7-methyl -5H-thiazolo- [3,2-a] pyrimidine-5-one), was found to inhibit diacylglycerol kinase in human red blood cell membrane and in intact platelets (de Chaffoy de Courcelles, et al., 1985). It has been shown also, to potentate the secretion and aggregation responses in thrombin stimulated platelets (Nunn & Watson 1987), and enhance superoxide generation from stimulated neutrophils (Muid et al., 1987). Accordingly, this diacylglycerol kinase inhibitor was used to test its effect on BHK cells and mouse pulmonary B10.D2 endothelial cell adhesion to polystyrene TC dishes.

i. The effect of diacylglycerol kinase inhibitor (R59022) on BHK cell adhesion.

The effect of the diacylglycerol kinase inhibitor was first examined on BHK cell adhesion in both 3% serum Ham's F-10 and in serum-free Ham's F-10 medium.

In the first experiment, BHK cells were trypsinised and 3%

serum Ham's F-10 used to stop tryptic activity and set at 0.2 x 10^6/ml. Different concentrations of diacylglycerol kinase inhibitor were added to the cells, incubated for 20 mins at 37°C and adherent cells were counted (Table.15).

In the second experiment leupeptin was used to stop trypsin activity and cells were resuspended in fresh serum-free Ham's F-10 medium. Then, BHK cells at a population density of 0.2 x 10^6/ml were incubated with diacylglycerol kinase inhibitor in tissue culture dishes for 20 mins at 37°C and cell adhering from suspension after this period were counted (Table.16). BHK cell adhesion in 3% serum Ham's F-10 was lower than the adhesion in serum-free conditions, in that 18.4 µg/ml of the inhibitor in serum Ham's F-10 were sufficient to reduce the adhesion to 39% of the control values, though 30 µg/ml did not decrease the adhesion to the same level in serum-free conditions.

ii. The effect of diacylglycerol kinase inhibitor (R59022) on B10.D2 endothelial cell adhesion.

In a similar experiment the effect of diacylglycerol kinase inhibitor on B10.D2 endothelial cell adhesion was studied. Confluent flask of B10.D2 were trypsinised and 3% serum Ham's F-10 medium was used to inactivate trypsin. The cells were resuspended in fresh 3% serum Ham's F-10 medium and used at 0.2x10^6/ml. Diacylglycerol kinase inhibitor at concentrations ranging from 4.6 µg/ml to 18.4 was added to the cells, the mixture was plated into polystyrene TC dishes and incubated for 20 mins at 37°C to allow cell-substratum interaction.

The adhesion was sharply reduced at concentration 18.4 µg/ml to 18 % of the control samples (Table.17; 18)

In conclusion, BHK cells were more resistant to the inhibitory effect of diacylglycerol kinase inhibitor in both serum and serum-

Table.15. Diacylglycerol kinase inhibitor (R59022) effect on BHK fibroblast cell adhesion to polystyrene TC dishes in 3 % serum Ham's F10 medium.

DAG kinase inhibitor (μg/ml).	Cells adherent per unit area ± Standard deviation
0.0 (control)	29 ± 4.86
4.6	31 ± 6.78
9.2	32 ± 5.07
18.4	11 ± 5.20 (***)
25.0	5 ± 2.74 (***)
30.0	3 ± 1.43 (***)

BHK cells at concentration of $0.2x10^6$/ml were first mixed with DAG kinase inhibitor in glass universals, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.16. Diacylglycerol kinase inhibitor (R59022) effect on BHK cell adhesion to polystyrene TC dishes in serum-free Ham's F10 medium.

DAG kinase inhibitor (μg/ml).	Cells adherent per unit area ±Standard deviation
0.0	55 ± 14.46
9.2	58 ± 8.37
18.4	58 ± 13.04
30.0	32 ± 12.79 (***)

Cells at concentration of $0.2x10^6$/ml were first mixed with DAG kinase inhibitor in glass universals, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C. Unattached cells were then washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.17. Diacylglycerol kinase inhibitor (R59022) effect on B10.D2 endothelial cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10 medium.

DAG kinase inhibitor (μg/ml).	Cells adherent per unit area ± Standard deviation
0.0	23 ± 11.87
4.6	24 ± 17.53
9.2	20 ± 16.16
18.4	3 ± 3.13 (***)

Cells at concentration of $0.2x10^6$/ml were first mixed with DAG kinase inhibitor in glass universals, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C. After that, unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements from four replicates ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.18. Diacylglycerol kinase inhibitor (R59022) effect on B10 D2 endothelial cell adhesion to polystyrene TC dishes in 3% serum Ham's F10 medium.

DAG kinase inhibitor (μg/ml).	Cells adherent per unit area ± Standard deviation
0.0	29 ± 8.05
11	23 ± 7.57
15	21 ± 7.18 (**)

Cells at concentration of $0.2x10^6$/ml were first mixed with DAG kinase inhibitor in glass universals, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C. After this period unattached cells were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements ± standard deviation.

**Differ significantly from the control *(t-test), P<0.01*

free conditions in comparison with endothelial cells. However, both types were appreciably reduced in adhesion.

The exact mechanism underlying the reduced cell adhesion is unclear. However, this effect might be mediated by the activation of protein kinase C as a result of diacylglycerol accumulation, or diacylglycerol could have been further hydrolyzed to monoacylglycerol which in turn will be further metabolized to release fatty acid because the phosphatidic acid pathway is blocked. The fatty acid released in this way might be the cause of the observed decrease in adhesion.

3.2.2-2. Inositol trisphosphate effect on cell adhesion.
Experiments were carried out with inositol 1,4,5-trisphosphate (IP_3) a second messenger released as a result of the hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) in response to a wide variety of external stimuli. It increases intracellular calcium by mobilizing internal calcium stores.

In the first experiment 3% serum Ham's F-10 rather than leupeptin was used to stop tryptic activity. A chosen concentrations of IP_3 were added to BHK (Table.19) or B10.D2 endothelial cells (Table.20), plated into polystyrene TC dishes and incubated at 37°C. Dishes were removed after 20 mins incubation and adherent cells were counted. The adhesion was reduced by approximately 30% of the control at 20μM concentration (Table.19 & 20). The effect could be due to an increase in intracellular calcium. As it was mentioned before, IP_3 stimulates calcium release from intracellular stores through receptors is believed to reside in smooth endoplasmic reteculum. Therefore, after trypsinization of cells, IP_3 might have entered the cells, and hence causing an increase in intracellular calcium that somehow led to a reduced adhesion.

Table.19. The effect of inositol trisphosphate (IP_3) on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F10 medium.

IP_3 concentration (μM).	Cells adherent per unit area ± Standard deviation
0.0 (control)	28 ± 9.73
10	17 ± 7.66 (***)
20	18 ± 6.61 (***)

BHK cells were set at concentration of $0.2x10^6$/ml in 3% serum Ham's F-10. Then, IP_3 was added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C to allow cell-substratum adhesion. Non-adherent cells were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 30 measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.20. The effect of inositol trisphosphate (IP_3) on B10 D2 endothelial cell adhesion to polystyrene TC dishes in 3% serum Ham's F10 medium.

IP_3 concentration (μM).	Cells adherent per unit area ± Standard deviation
0.0	35 ± 13.34
10	34 ± 6.96
20	24 ± 8.89 (***)

Cells were set at concentration of $0.2x10^6$/ml in 3% serum Ham's F-10. Then, IP_3 was added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. After this period unattached cells were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

The above assumption was further investigated by preincubating the cells in serum or serum-free medium to recover from any trypsin effect before the addition of IP_3.

BHK cells were preincubated for 60 mins at 37°C in serum-free Ham's F-10 medium (Table.21, 22), or 3% serum Ham's F-10 medium (Table.23). After this period, IP_3 was added to the cells and incubated in polystyrene TC dishes for 20 mins at 37°C. Unattached cells were washed off and adherent cells were counted.

The adhesion was reduced by 20 to 24% of the control at 30 and 40μM. No such effect was found with 20μM IP_3, a concentration which reduced significantly ($P<0.001$) the adhesion of non-preincubated cells. This might suggest that the trypsinization was to some extent responsible for the effect of IP_3 on cell adhesion. However, high concentrations of IP_3 are still reducing the adhesion of preincubated cells. In this case, IP_3 could have exerted its effect without any need to cross the plasma membrane.

It was already suggested that external IP_3 could induce in intact cells an influx of external calcium through transmembrane channels (Michell 1975; Kuno and Gardner 1987).

I next sought to investigate the binding of IP_3 by intact BHK cells. In this experiment, $3x10^7$ BHK cells were incubated with different concentrations of ^{3}-H-IP_3 for 15 mins at 37°C. Then, cells were washed and the radioactivity in cells and the wash was counted. Results in Fig.(4.9 a&b), show that approximately 0.3% of the total IP_3 used was bound by the cells. This corresponds to about $27x10^3$ molecules per cell.

3.2.2-3. The effect of Phorbol myristate acitate (PMA) and ionomycin on BHK cell adhesion.

Phorbol myristate acetate and ionomycin are known for their

Table.21. The effect of inositol trisphosphate (IP_3) on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F10 medium. (preincubation of cells for 60 mins in serum-free Ham's F-10 after trypsinization)

IP_3 concentration (μM).	Cells adherent per unit area ± Standard deviation
0.0 (control)	36 ± 7.08
5	36 ± 18.22
10	39 ± 13.34
20	30 ± 17.37

BHK cells at concentration of $0.2x10^6$/ml were first preincubated for a period of 60 mins at 37°C in serum-free Ham's F-10 prior to the addition of IP_3 (see text for relevance). Then, IP_3 was added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C to allow cell-substratum adhesion. Non-adherent cells after that were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements ± standard deviation.

Table.22. The effect of higher concentrations of inositol trisphosphate (IP_3) on BHK fibroblast cell adhesion to polystyrene TC dishes in serum-free Ham's F10 medium.

IP_3 concentration (μM).	Cells adherent per unit area ± Standard deviation
0.0	42 ± 8.04
20	38 ± 7.95
30	33 ± 5.30 (***)
40	34 ± 5.87 (***

BHK cells at concentration of $0.2x10^6$/ml were first preincubated for a period of 60 mins at 37°C in serum-free Ham's F-10 prior to the addition of IP_3 (see text for relevance). Then, IP_3 was added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells were then washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.23. The effect of inositol trisphosphate (IP_3) on BHK fibroblast cell adhesion to polystyrene TC dishes in 3% serum Ham's F10 medium. (preincubation of cells for 60 mins in 3% serum Ham's F-10 after trypsinization)

IP_3 concentration (μM).	Cells adherent per unit area ± Standard deviation
0.0 (control)	34 ± 6.71
20	29 ± 6.74
40	26 ± 6.03 (***)

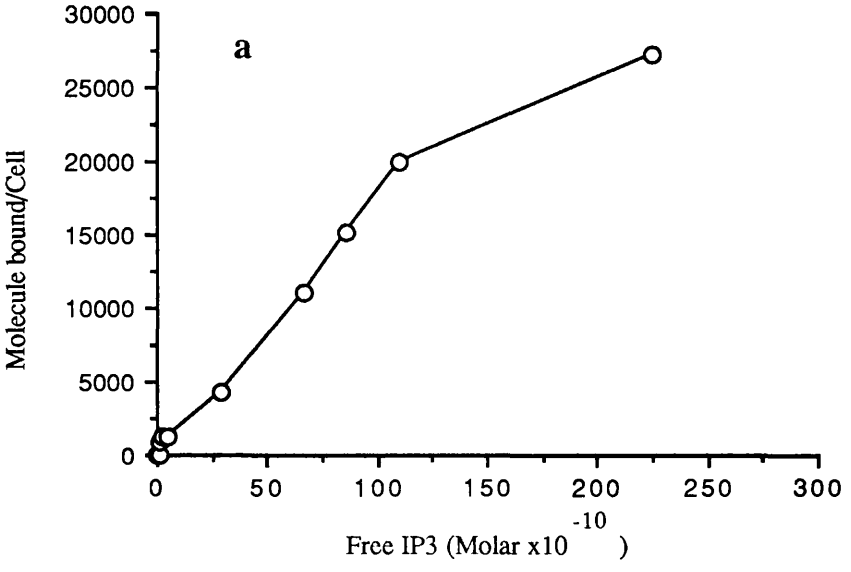
BHK cells at concentration of $0.2x10^6$/ml were first preincubated for a period of 60 mins at 37°C in 3% serum Ham's F-10 prior to the addition of IP_3 (see text for relevance). Then, IP_3 was added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 20 mins at 37°C. Unattached cells were afterward washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $2.5x10^{-3}$ cm^2. Means of 40 standard measurements ± standard deviation.

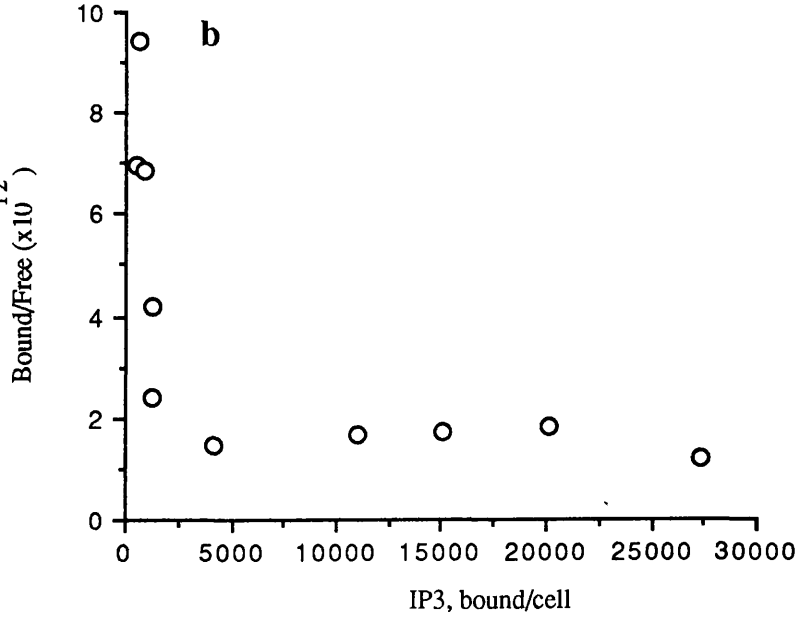
***Differ significantly from the control *(t-test), P<0.001.*

Fig.4.9. The binding of ^{3}-H-IP_3 to BHK cells in serum-free medium.

Cells were trypsinised in usual manner, leupeptin used to stop trypsin activity, resuspended in fresh serum-free Ham's F-10 medium and set at 10 x10^6 /ml in plastic universals and kept on ice. 3ml 10 x10^6 /ml cells were then added to each of 10 plastic universals and ^{3}-H-IP_3 (specific activity 1 Ci/mmol) was added to the cells at different concentrations (9.76x10^{-13} - 5x10^{-10} moles). Cells were after that incubated for 10 mins at 37°C and the ^{3}-H-IP_3 bound to the cells as well as the free were counted.

a. Direct plot. Free IP_3 (molar) is plotted versus molecules of IP_3 bond per cell.
b. Scatchard plot. In curve (b) molecules of IP_3 bound per cell are plotted versus bound divided by the free IP_3.


a
30000
25000
20000
15000
10000
5000
0
Molecule bound/Cell
0
50
100
150
200
250
300
-10
Free IP3 (Molar x10)


b
10
8
6
4
2
0
12
Bound/Free (x10)
0
5000
10000
15000
20000
25000
30000
IP3, bound/cell

synergistic effect in affecting many cellular activities including secretion, adhesion, aggregation and proliferation of many cell types. PMA can bypass the signal transduction pathway and activate Protein kinase C in a direct fashion, while ionomycin increases cytoplasmic free calcium levels.

In assessing a role for protein kinase C in BHK fibroblast cell adhesion, PMA and ionomycin were tested.

A dose response experiment was carried out with both PMA (1-4 μg/ml) (Fig.4.10) and ionomycin (1-8 μM) (Fig.4.11) to test their effect on BHK cell adhesion to polystyrene TC dishes in 3% serum Ham's F-10. A small increase in the adhesion after 20 mins incubation at 37°C was observed at all concentrations used in the case of PMA, whereas, no significant alteration in the adhesion was seen in the case of ionomycin. Ionomycin (1-16μM) effects on BHK cell adhesion in very low calcium medium (see methods) were also examined (Fig.4.12 a&b). In another experiment, the possible synergistic effect of PMA and ionomycin on BHK cell adhesion was tested. PMA and ionomycin were added singly or in mixture to the cells and the number of cells adherent to polystyrene TC dishes was counted after 10 mins incubation at 37°C (Fig.4.13). Though PMA added alone to the cells increased cell adhesion, no clear evidence for synergism was seen with ionomycin under the above conditions.

3.2.2-4. The effect of PMA and PGE_1 on BHK cell and PMN leukocyte adhesion.

The co-effect of PMA (10 ng/ml) and PGE_1 (5-100μM) on BHK adhesion to polystyrene TC dishes was tested in 20 % serum containing medium, and compared with human PMN leukocyte adhesion under the same conditions.

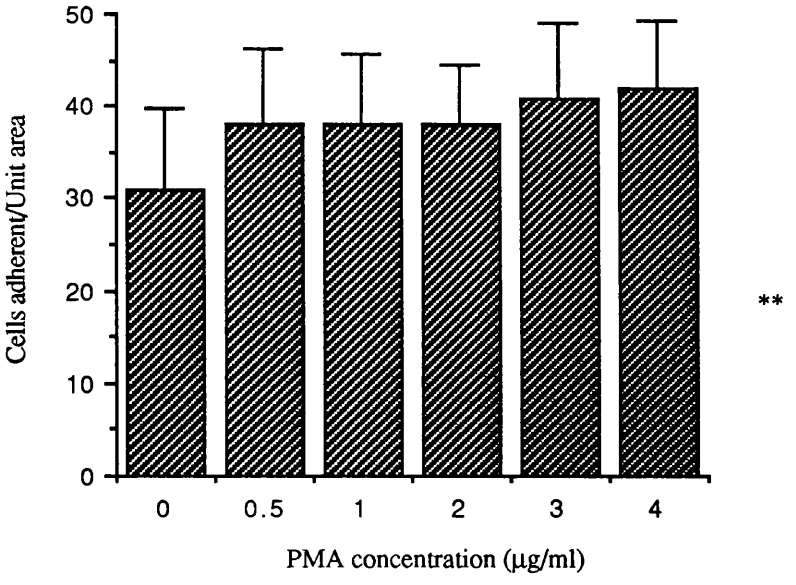


Fig.4.10. Dose response histogram of PMA effect on BHK cell adhesion to polystyrene dishes in 3% serum Ham's F-10 medium.

BHK cells were first trypsinised, 5 ml 3% serum Ham's F-10 used to neutralize trypsin activity, span, resuspended in fresh 3% serum Ham's F-10 medium and set at $0.2x10^6$/ml. PMA was then added to the cells and 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

** All concentrations differ significantly from untreated sample *(t-test), $P< 0.01$.*

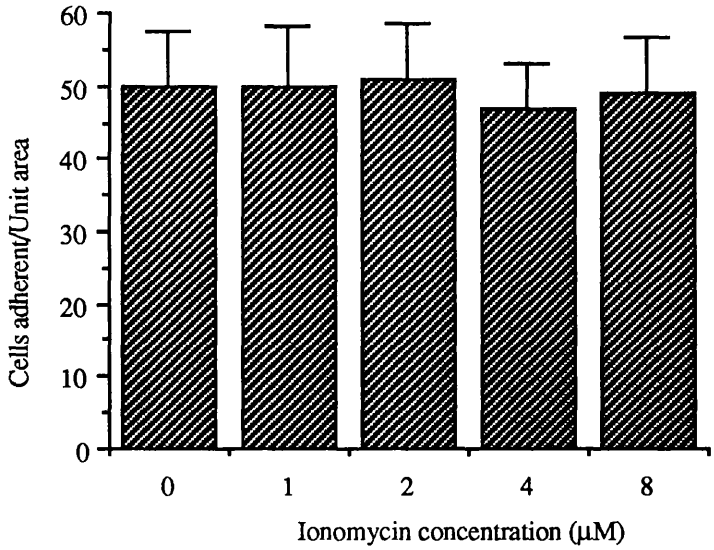


Fig.4.11. Dose response histogram of Ionomycin effect on BHK cell adhesion to polystyrene dishes in 3% serum Ham's F-10 medium.

BHK cells were first trypsinised, 5 ml 3% serum Ham's F-10 used to neutralize trypsin activity, span, resuspended in fresh 3% serum Ham's F-10 medium and set at 0.2×10^6/ml. Ionomycin was then added to the cells at final concentration of 1; 2; 4; and 8 μM. 3 ml (0.2×10^6/ml) cells were plated into each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of 3.25×10^{-3} cm^2. Means of 30 standard standard measurements. Error bars are one standard deviation.

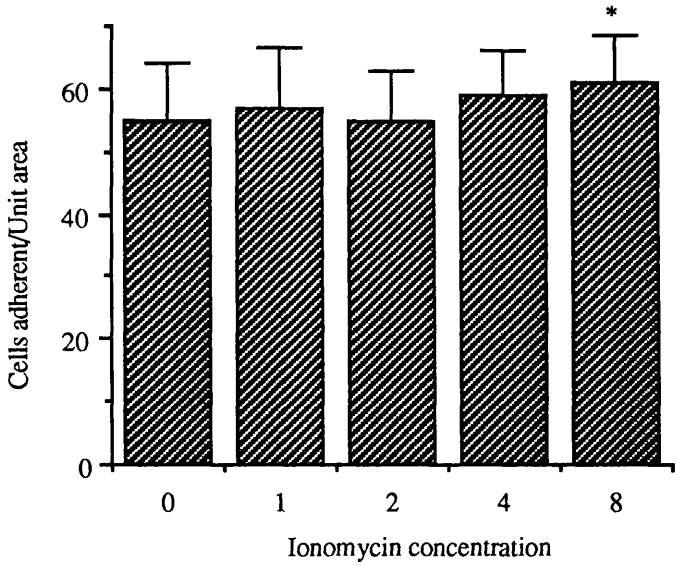


Fig.4.12a. Dose response histogram of ionomycin effect on BHK cell adhesion to polystyrene dishes in serum-free (low Ca^{2+}) medium.

5 ml 3% serum Ham's F-10 used to stop trypsin activity, washed twice with low calcium medium, resuspended in fresh low calcium medium and set at 0.2 $x10^6$ /ml in glass universals. Ionomycin was then added to the cells at final concentration of 1; 2; 4; and 8 µM. 3 ml ($0.2x10^6$/ml) cells were plated into each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

* Differ significantly from untreated sample *(t-test), P< 0.05.*

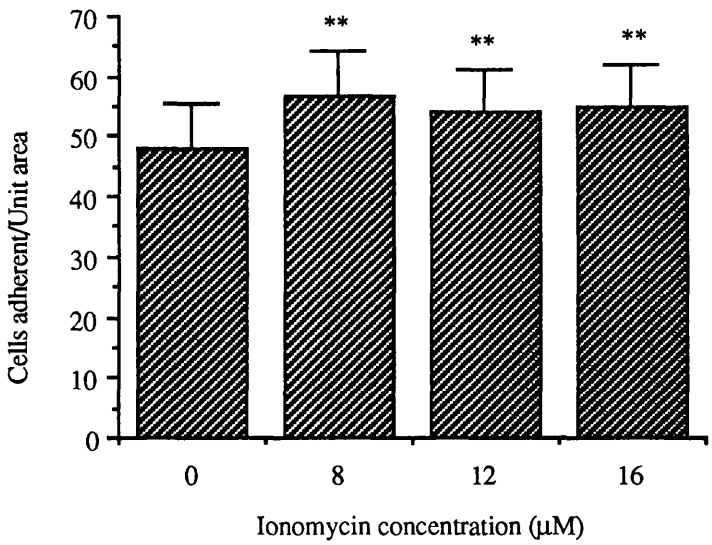


Fig.4.12b. Ionomycin (higher concentrations) effect on BHK cell adhesion to polystyrene dishes in serum-free (low Ca^{2+}) medium.

5 ml 3% serum Ham's F-10 used to stop trypsin activity, washed twice with low calcium medium, resuspended in fresh low calcium medium and set at 0.2 $x10^6$ /ml in glass universals. Ionomycin was then added to the cells at final concentration of 8; 12; and 16 μM. 3 ml ($0.2x10^6$/ml) cells were plated into each polystyrene TC dish and incubated for 20 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

** Differ significantly from the control *(t-test), P< 0.01 respectively).*

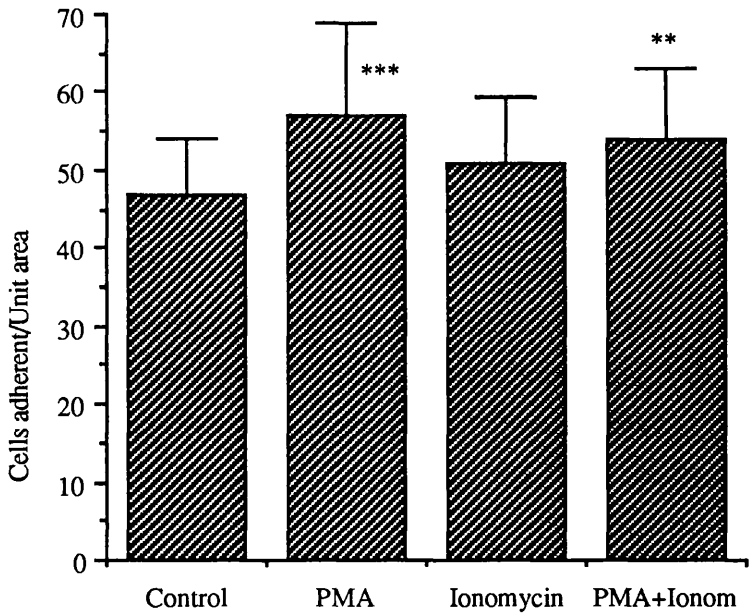


Fig.4.13. The synergistic effect of PMA and Ionomycin on BHK cell adhesion to polystyrene dishes in 3% serum Ham's F-10 medium.

Cells were trypsinised in usual manner, 5 ml 3% serum Ham's F-10 used to stop trypsin activity, resuspended in fresh 3% serum Ham's F-10 medium and set at $0.2x10^6$/ml in glass universals. Ionomycin (1μM) and PMA (2μg/ml) was then added singly or in mixture to the cells. 3 ml ($0.2x10^6$/ml) cells were plated into each polystyrene TC dish and incubated for 10 mins at 37°C to allow cell-substratum adhesion. Unattached cells after this period were gently washed out and adherent cells counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements. Error bars = one standard deviation.

***, ** Differ significantly from the control *(t-test), P< (0.001, 0.01 respectively).*

i. BHK cells.

BHK cells incubated for 30 mins at 37°C with 10 ng/ml PMA and different concentrations (1-100μM) of prostaglandin E_1 did not show any alteration in their adhesion (Table.24). However, the spreading area increased significantly (Table.25). PMA (10 ng/ml) increased the mean spreading area per cell when added by it self but not to the same extent as it was seen with PMA and PGE_1 together.

A dose response experiment was carried out with PGE_1 in 20% serum-containing medium. In this experiment PGE_1 was added by itself using different concentrations. There was a slight enhancement of BHK cell adhesion to polystyrene TC dishes (Table.26).

ii. PMN leukocytes.

PMN leukocytes incubated for 30 mins at 37°C with PMA or PMA (10 ng/ml) and PGE_1 show a significant increase in their adhesion to polystyrene TC dishes (Table.27) in comparison with PMN base line adherence. It is clear that PMA significantly increased PMN base line adherence and 50 μM PGE_1 inhibited PMN leukocyte adhesion stimulated with PMA, but not the base line adherence, (unstimulated) (see Table. 28).

3.2.2-5. Morphological changes.

BHK cells incubated with PMA (4μg/ml) or ionomycin (4μM) display a morphology that is clearly different from that of untreated samples (Plate. 8). After few hours (6-9 hours) of incubation they start to elongate and became needle-like shaped with a significant reduce in their volume. This morphology disappear however afterward, when cells were further incubated in fresh medium (plate.8). In fact PMA-treated BHK cells were able to recover from PMA effect without

Table.24. Co-effect of PGE_1 and PMA on BHK cell adhesion to polystyrene TC dishes in 20 % serum medium.

Samples.	Cells adherent per unit area ± Standard deviation
Control	48 ± 8.45
Control with PMA only (10 ng/ml)	49 ± 8.92
1 μM PGE_1+ 10 ng/ml PMA	49 ± 8.41
5 μM PGE_1+ 10 ng/ml PMA	45 ± 8.38
10 μM PGE_1+ 10 ng/ml PMA	48 ± 10.22
50 μM PGE_1+ 10 ng/ml PMA	44 ± 9.82
100 μM PGE_1+ 10 ng/ml PMA	44 ± 6.84

PGE_1 (1-100 μM) and/or PMA (10 ng/ml) were added to the cells, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 30 mins at 37°C in 20 % serum MEM medium. Unattached cells were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

Table.25. Co-effect of PGE_1 and PMA on BHK cell spreading on polystyrene TC dishes in 20 % serum medium after 30 mins incubation at 37°C.

Samples	Mean spreading area/cell (μm^2)	
Control	209 ± 144.50	
Control with PMA only (10 ng/ml)	348 ± 302.56	
1 μM PGE_1+ 10 ng/ml PMA	374 ± 351.94	
5 μM PGE_1+ 10 ng/ml PMA	383 ± 280.02	
10 μM PGE_1+ 10 ng/ml PMA	383 ± 298.35	
50 μM PGE_1+ 10 ng/ml PMA	418 ± 372.94	
100 μM PGE_1+ 10 ng/ml PMA	316 ± 221.27	***

PGE_1 (1-100 μM) and/or PMA (10 ng/ml) were added first to the cells, then, 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for 30 mins at 37°C in 20 % serum MEM medium. After that, unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and the area of each adherent cell was then measured. Cells were visualized with x10 objective and spreading is expressed as mean spreading area per cell (μm^2) ± standard deviation. More than 500 cells were measured for each experimental condition in three replicate TC dishes.

***All values differ significantly from the control *(t-test), P<0.001.*

Table.26. PGE_1 effect on BHK cell adhesion to polystyrene TC dishes in 20 % serum medium.

PGE_1 concentration (μM).	Cells adherent per unit area ± Standard deviation
Control	48 ± 10.74
1	55 ± 9.95
5	52 ± 11.31
10	62 ± 9.57 (***)
50	57 ± 10.89 (***)

PGE_1 (1-100 μM) was added first to the cells, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 30 mins at 37°C in 20 % serum MEM medium. After this period unattached cells were washed with HS, and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

***Differ significantly from the control *(t-test), P<0.001.*

Table.27. Co-effect of PGE_1 and PMA on PMN leukocyte adhesion to polystyrene TC dishes in 20 % serum medium.

Samples	PMN adherent per unit area ± Standard deviation
Control (unstimulated)	27 ± 6.17
Control with PMA only (10 ng/ml)	58 ± 8.05
1 μM PGE_1+ 10 ng/ml PMA	51 ± 7.28 (**)
5 μM PGE_1+ 10 ng/ml PMA	51 ± 9.44 (**)
10 μM PGE_1+ 10 ng/ml PMA	49 ± 8.78 (***)
50 μM PGE_1+ 10 ng/ml PMA	48 ± 9.54 (***)

PGE_1 (1-50 μM) and/or PMA (10 ng/ml) were added first to PMN leukocytes in 20% serum MEM medium, then 3 ml ($0.2x10^6$/ml) cells were added to each polystyrene TC dish and incubated for a period of 30 mins at 37°C. After this period unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

,*Differ significantly from control with PMA *(t-test), P<0.01 and 0.001 respectively.*

Table.28. PGE_1 effect on PMN leukocyte adhesion to polystyrene TC dishes in 20 % serum medium.

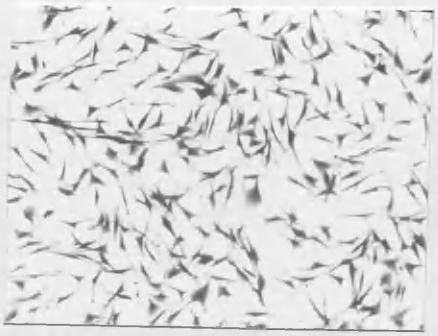
PGE_1 concentration (μM).	Cells adherent per unit area ± Standard deviation
Control (unstimulated)	36 ± 5.99
1	39 ± 7.35
5	37 ± 7.03
10	37 ± 7.94
50	40 ± 9.97

PGE_1 (1-50 μM) was added to PMN leukocytes, then 3 ml ($0.2x10^6$/ml) PMN leukocytes were added to each polystyrene TC dish and incubated for a period of 30 mins at 37°C in 20 % serum MEM medium. After that, unattached cells were washed with HS and the adherent cells were fixed with formal saline, stained with kenacid blue and then counted. Adhesion is expressed as number of cells adherent per unit area of $3.25x10^{-3}$ cm^2. Means of 30 standard measurements ± standard deviation.

Plate. 8. Morphological change of BHK cells induced by PMA or ionomycin.
BHK cells were incubated at 37°C in polystyrene tissue culture dishes in 3% serum ham´s F-10 only (a), with PMA (b) or ionomycin (c). They were then removed after 12 hours (1),or 72 hours (2)*,then,fixed stained and photographed.

*The medium was changed after approximately 48 hours.

Scale bar = 200 μm.

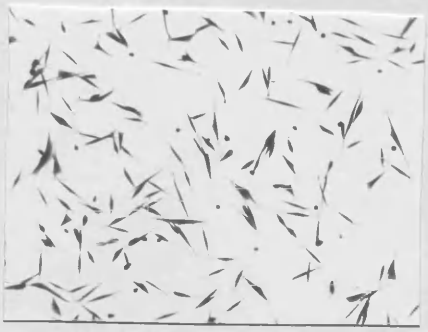


a_1

a_2

b_1

b_2



c_1

c_2

changing the incubation medium. This may probably suggest that BHK cells have depleted the PMA in the medium by metabolic degradation. The morphology of PMA or ionomycin treated cells (after recovering from PMA or ionomycin effect), is still unlike control cells.

PGE_1 was also found to modulate BHK cell shape. Cells incubated for a period of 65 mins at 37°C with 10 or 100 μM PGE_1 in 3% serum Ham´s F-10, led to a change in their shape which included the appearance of process like growth and flattening (plate.9). It is clearly apparent that unlike control cells which appear in fibroblastic shape, treated cells show an increase in the number of narrow cytoplasmic processes which made them star-like shaped. This type of effect was shown to be mediated by an increase in cytoplasmic cAMP especially in cells of neuronal origin such as PC12. This suggest that PGE_1 may have stimulated adenylate cyclase and therefore increased intracellular cAMP which in turn might have affected many cellular structures that led to the observed effect.

It is clear from this section that BHK fibroblast cell adhesion, spreading and cell morphology can be modulated using agents known to activate many cellular functions in other cell types in particular blood cells. Therefore activation of cell functions could be a wide spread phenomenon for many cell types including fibroblastic cells.

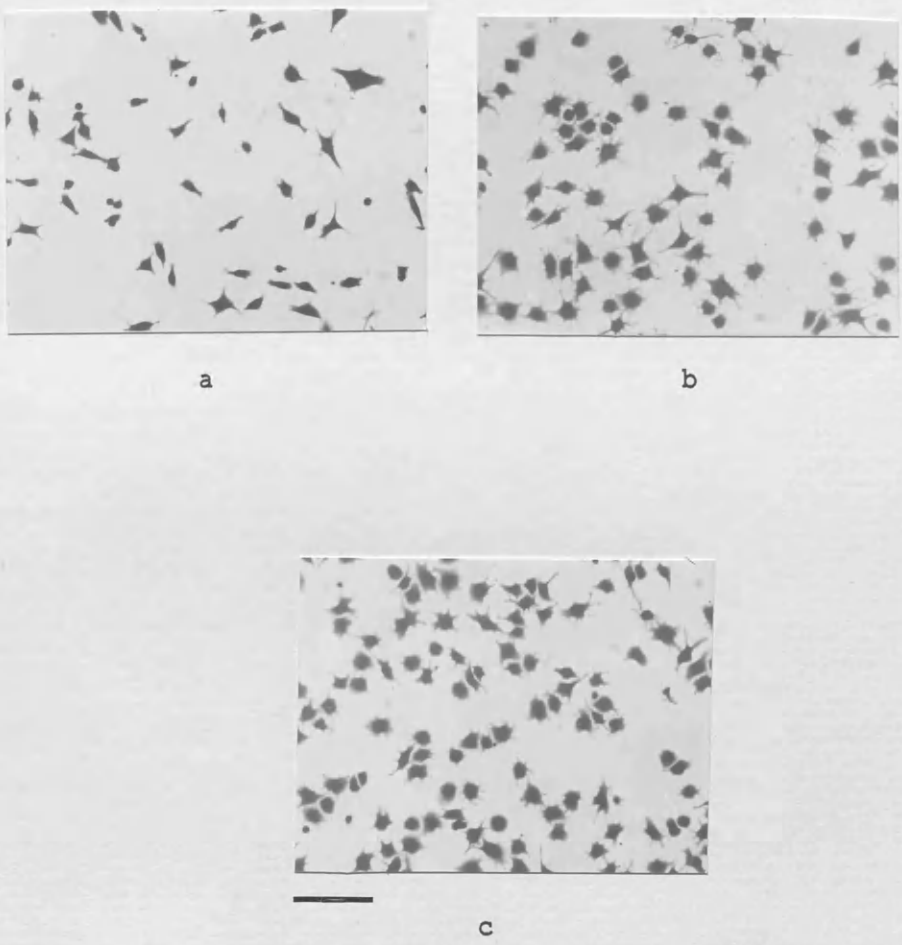


Plate.9. Morphological change of BHK cells induced by PGE_1. Cells were incubated at 37°C with PGE_1 in polystyrene tissue culture dishes. They were then removed after 65 mins, fixed, stained and photographed under an inverted microscope.

a. Control, b. 10 μM PGE_1 and c. 100 μM PGE_1.

Scale bar = 100 μm.

Chapter IV
Discussion

Chapter IV. Discussion.

The aim of this work is on one hand an extension to previous work that showed a correlation between plasma membrane phospholipids modulation and cell adhesion and on the other hand an attempt to see whether BHK fibroblasts have activation events like leukocytes. The two topics are closely related, since activation events often involve lipid modulation. However, because of the division of the Result's chapter to two sections, the discussion of the results is divided into two sections as well.

Section 4.1. Membrane phospholipids modulation and BHK cell adhesion.

The membrane fatty acids can influence many membrane properties including fluidity, flexibility, behaviour of membrane-bound proteins such as receptors, enzymes and ion channels (see Introduction).

It is known from previous work (Fischer et al., 1967; Horwitz et al., 1974; Curtis et al., 1975c; Schaeffer and Curtis 1977, etc.) that the lipid composition of the plasmalemma can be altered by taking advantage of its own acyltransferase system to incorporate appreciable amounts of a selected fatty acid into plasmalemmal phosphatidyl-lipids. It has also been shown that such lipid change can be used to alter cell adhesion in the neural cells of the chick retina (Curtis et al., 1975 abc) as well as in tissue culture cells such as Chinese hamster ovary, baby hamster kidney (BHK) and Mouse L929 fibroblastic cells (Schaeffer and Curtis 1977; Hoover et al., 1977). Therefore, I have extended earlier work by:

i) using fatty acids in the acyl-CoAs form; ii) different methods and systems for measuring cell-substratum adhesion were also used in addition to the same methods as in earlier work; iii) the adhesion was measured in serum-containing as well as serum-free

medium; iv) leupeptin (a protease inhibitor), rather than serum containing medium, was used to stop trypsin activity in experiments designed to be in serum-free conditions.

Because of the effects observed on cell adhesion and spreading when cells were treated with acyl-CoAs or fatty acids, the experimental results obtained in ***section.3.1,*** suggest in general that the chemical as well as physical state of plasma membrane can control many physiological functions including the above functions. Though some of the acyl-CoAs (linolenoyl-CoA and arachidonoyl-CoA) used did not affect the above functions, others, such as stearoyl-CoA, oleoyl-CoA had a clear effect on the adhesion (increase at low concentrations and reduction at high concentrations), spreading (decrease) and viability (reduced at high concentrations) of BHK cells. The incorporation data suggests that the observed effect on cell adhesion and spreading could be due to an alteration in plasma membrane properties that include fluidity, deformability and intramembrane electrodynamic forces. Since a relatively large quantity of the fatty acid incorporated is intracellularly, other effects, cannot be excluded. These include intracellular metabolism of the fatty acid, an effect on intracellular membrane properties or other structures. However, I did not study the incorporation of all fatty acids or acyl-CoAs used directly in adhesion studies and therefore I have to rely to some extent on the work of others such as Hoover et al., (1977).

The main findings in section 3.1 were as follow:

1. The long chain stearoyl-CoA and oleoyl-CoA were found to affect cell adhesion in serum-free medium in two similar ways. A significant increase in cell adhesion at low concentrations (1.75-7 μM) and a decrease at fairly high concentrations (14-28 μM). Both stearoyl-CoA

and oleoyl-CoA at concentrations ranging between 1.75-28 μM, reduced significantly the spreading area of cells.

2. The unsaturated acyl-CoAs (linoleoyl-, linolenoyl- and arachidonoyl-CoA) however, have little or no effect on the adhesion and the spreading of cells at any concentration used. Perhaps this implies that the normal composition of the plasmalemma includes these acyl groups at about the same proportions, so that substitution has not appreciably altered the membrane.

3. All the above acyl-CoAs used did not however alter cell adhesion, spreading or viability at all in the presence of serum-containing medium. The results are interesting since there has been a tendency to equate adhesion and spreading, but in 1, the adhesion rose as spreading fell.

4. Arachidonic and linoleic acid used in the free acid form decreased the adhesion sharply and reduced the viability in serum and in serum-free conditions, particularly at high concentrations. In serum-free media the viability was as low as 2% when 10 μg/ml and higher concentrations of the arachidonic acid were used. In contrast, no loss of viability was seen when arachidonic acid was used at 10 mg/ml and lower concentrations in serum-containing medium. Linoleic acid however, reduced the viability to approximately the same extent (less than 5%) in both serum and serum-free conditions, when a concentration of 10 μg/ml and higher was used. This indicates that the reduced cell adhesion is due to the loss of viability caused by the use of the above fatty acids.

5. BHK cells after 20 minutes incubation at 37°C in serum or serum-free media supplied with acyl-CoA, incorporated a substantial amount (3-8%) of the exogenous fatty acyl-CoA into their plasma membrane, in particular into their lipid components.

---

1. Explaining the effect of stearoyl-CoA and oleoyl-CoA.
The mechanism underlying the effect of stearoyl-CoA and oleoyl-CoA on BHK cell adhesion is unclear. However, there are some possible explanations:

i. An alteration in the lipid thermal transition temperatures of plasmalemmal phosphatidyl compounds in which fatty acid substitution were made (Horwitz et al., 1974, Williams et al., 1974), affecting lipid fluidity and thus the mobility of adhesion receptors.

ii. A change in membrane electrodynamic forces (van der Waals) that results from the change in fatty acid composition of the plasma membrane (Curtis, 1973; Curtis et al., 1975c), and therefore, changing the dielectric constant (see Jones, 1974).

iii. Metabolic conversion to prostaglandins and leukotrienes.

---

i. In the case of fluidity, the saturated fatty acids would exert a solidifying effect (decrease in fluidity) on the membrane at experimental temperatures. The reverse would be expected to occur when unsaturated fatty acids are incorporated into the plasma membrane, provided that the membrane has an equal proportion of saturated and unsaturated fatty acids. For instance, chick embryonic plasmalemmal lipids appear to be mainly a stearoyl- in position 1 and oleoyl- in position 2.

The precise fluidity of the plasma membrane must be biologically important, since bacteria, yeast and other poikilothermic organisms, whose body temperatures fluctuate with that of their environment, change the fatty acid composition of their plasma membrane to

maintain a relatively constant fluidity. As temperature falls, for example, fatty acids with more cis-double bonds are synthesized to avoid the decrease in bilayer fluidity that would result from the drop in temperature.

The functions that might be affected when changing membrane fluidity are the insertion and distribution of proteins, the lateral movement of molecules within the plane of the membrane, membrane thickness and the activity of membrane-bound enzymes. These molecular events might be of great relevance to cell adhesion.

Therefore, changes in membrane fluidity might affect adhesion by altering the distribution of any membrane sites that might be involved in cell adhesion (Schaeffer and Curtis 1977).

It is already known that the plasma membrane fluidity can be altered by supplying cells with selected fatty acids or lipids (see Schaeffer and Curtis, 1977).

Both stearoyl-CoA, and oleoyl-CoA (the later unsaturated in only one position), might have exerted a solidifying effect on the membranes by the fact that they might have been reacylated into positions previously occupied by poly-unsaturated fatty acids of the phospholipid molecules and therefore reducing the lateral mobility of molecules. Therefore, the increase in viscosity might have maintained the adhesive sites in stable cluster which led to enhanced adhesion. The reduced cell spreading might be related also to the decrease in viscosity. A fluid membrane is more flexible than a viscous one. Therefore, the more fluid the membrane is, the more easily the cell can spread and hence, the incorporation of saturated or mostly saturated fatty acyl-CoA into plasma membrane would be expected to exert a solidifying effect and therefore a less flexible membrane.

It was shown that red cell-membranes deficient in essential fatty acids become stiffer than normal and do not pass through capillaries

as readily (Simpson, 1988). Hawley and Gordon (1976) showed that oleic and palmitic acid inhibited neutrophil chemotaxis in a dose-dependent manner. They indicated also that saturated fatty acids caused a far greater inhibition of chemotaxis than the unsaturated fatty acids. They explained this effect in terms of the presence of clefts in the endoplasmic reticulum which they presume to be crystals of fatty acids or triglyceride which may have mechanically inhibited chemotaxis by decreasing deformability of the cells.

It was shown that there is a correlation between membrane fluidity and the critical temperature for cell adhesion. Ueda et al., (1974) found that the adhesion of BHK cells is temperature-dependent and the critical temperature for cell adhesion coincided well with one of the characteristic temperatures for the membrane fluidity change.

The incorporation of polyunsaturated fatty acids (linoleic, linolenic and arachidonic acid) into the slime mold *Dictyostelium discoideum*, was shown to alter cell-cell contacts which in turn affects differentiation (Weeks, 1976). Horwitz et al., (1974), showed that exogenously supplied fatty acids are incorporated and alter the fatty acid composition of membrane phosphatide which in turn affected growth, agglutination, and cell morphology.

Staltz et al., (1973) reported that platelets aggregate more rapidly in the presence of long chain saturated fatty acids and progressively less rapidly with an increase in the number of double bonds per molecule. Prives and Shinitzky, (1977), have observed that exposure of myoblasts to fatty acids that increase membrane microviscosity retarded fusion. Conversely, treatment of cells with fatty acids that decrease membrane viscosity facilated fusion.

ii. Concerning electrodynamic forces, is that by altering the chain length and unsaturation of the plasma membrane phospholipids,

intramembrane dynamic forces which could play a role in adhesion (Curtis, 1967, 1973) might be affected. Electrodynamic as well as electrostatic forces are amongst the determinants of the dielectric constant of the cell surfaces. Hence, the hydrocarbon chain length and degree of saturation of the fatty acid components of cell surface lipids may play an important role in determining the value for the dielectric constant.

The modification of the dielectric constant of surfaces or immersion medium may provide a strong test for the action of long range attractive forces in adhesion. Jones, 1974, observed a steady increase in the adhesive interaction of cells with increasing the dielectric constant of the medium. The observed effect of stearoyl-CoA and oleoyl-CoA on adhesion might be due to a modification of the cell surface dielectric constant, though the extent of change is likely to be small.

iii. Stearoyl-CoA and oleoyl-CoA could have been further desaturated (Williams et al., 1974) and used for eicosanoids synthesis. However, the results obtained with arachidonoyl-CoA (see below) the precursor molecule for eicosanoids synthesis, indicate that the effects of stearoyl-CoA and oleoyl-CoA on BHK cell adhesion and spreading might discount a mechanism involving arachidonic acid metabolism.

Other effects cannot be excluded, which include effects on enzyme activity, protein acylation, intracellular degradation of the fatty acyl-CoAs used, etc. All these matters could be tested in further work.

2. The inability of polyunsaturated fatty acyl-CoAs, in particular arachidonoyl-CoA and linoleoyl-CoA acid to affect the adhesion in serum-free conditions could be due to the following:

Cells might have regulated their membrane. Cholesterol in particular, which is known to have a rigidifying effect on the membrane (Oldfield & Chapman 1972) may have returned the membrane fluidity to a normal level.

It could be argued also that unsaturated fatty acids are usually in the second position of phospholipids and are a prime target for PLA_2 activity. This might suggest that the substitution (exchange) of an unsaturated fatty acid with another unsaturated fatty acid within the plasma membrane would not affect cell function.

The acyl-CoAs used could be the normal constituent of BHK cell membrane lipids. In this respect, Weeks, (1976), indicated that the growth of *Dictyostelium discoideum* in media containing polyenoic fatty acids that are not normal constituent of its membrane lipids produced a dramatic alteration in the fatty acid composition of the cells.

Unsaturated fatty acids might have inactivated PLA_2 (Ballou and Cheung, 1985), so there would be no turnover. It was found that unsaturated fatty acids inhibited platelet PLA_2 activity by 50% (Ballou and Cheung, 1985).

Another possible explanation is that this fatty acids might have been a favorable substrate for metabolic conversion once incorporated. It is known that subsequent metabolism of the incorporated fatty acid depends upon the nature of the fatty acid provided (Horwitz et al., 1974).

3. The acyl-CoAs used did not alter cell adhesion, spreading or viability at all in the presence of serum-containing medium.

The inability of these acyl-CoAs to affect the above functions might be due to the presence of either an acyl binding agent in the serum, the presence of an inhibitor or because lipids are present in

the medium which prevented the access of stearoyl-CoA to the membrane or because the cells have the same fatty acids composition to that provided in the medium. It is known that animal cells are normally grown in media containing serum and therefore derive almost all their lipids from the growth medium.

In fact, incorporation experiments (see below) performed in serum-containing medium revealed that there was a 43% reduction in the total uptake compared with incorporation carried out in serum-free conditions. This might clearly support the assumptions that there is a binding substance in the medium that have reduced the incorporation of the acyl-CoAs. The nature of this binding agent is unclear. It could be however, a lipid molecule or a protein. In this respect albumin is a very well known acyl-binding protein (Spector, 1975). Thus it could have been a competitive binding between the fatty acids, other organic compounds in serum that use albumin as a carrier protein and the added acyl-CoAs. Though, the albumin present in the serum could be saturated with fatty acids and other organic compounds. In the usual physiological concentration range, free fatty acids do not displace appreciable amounts of a second organic compound from albumin. A small increase in free fatty acid concentration however, might alter the molecular interaction between albumin and another organic substance (Spector, 1975).

This could be overcome in further work using albumin-depleted and/or delipidated serum medium or albumin saturated with specific fatty acids.

4. The effect of arachidonic and linoleic acid (also used as free form) on cell adhesion.

Unlike arachidonoyl-CoA and linoleoyl-CoA, arachidonic and linoleic acid added exogenously were found to decrease the adhesion

in a dose dependent manner. The viability was also found to be affected in similar fashion (see earlier). Thus, the observed effect on cell adhesion could be due to a loss in viability. However, the significant decrease in adhesion caused by arachidonic acid at low concentrations (2.5, 5μg/ml) in serum conditions could be explained in terms of fluidity effect, membrane electrodynamic forces change or the intracellular metabolism of arachidonic acid.

These results are in agreement with those of other workers who showed that arachidonic and linoleic acid decreased cell-cell (Curtis et al., 1975) and cell-substratum adhesion (Schaeffer and Curtis 1977; Hoover et al., 1977). They explained the effect in terms of change in intermembrane electrodynamic forces, alteration in membrane fluidity or a change in the membrane composition. These workers did not however, give a clear picture about the state of cells treated with fatty acids during the adhesion assays.

It was shown previously that at concentrations greater than 4 and 8 μg/ml respectively, linoleic and linolenic acids provided as Tween-esters were cytotoxic (Williams et al., 1974).

The ability of fatty acids in the free form unlike the acyl-CoA forms to influence cell functions including cell adhesion, is unclear. It could be argued however that the free form is readily taken up by the cells. Though the incorporation experiment shows clearly that a substantial amount of acyl-CoA was incorporated especially in serum-free medium. This might suggest that the mechanism of action is different. It should be mentioned that the control with ethanol shows that ethanol effect cannot be implicated.

5. Acyl-CoA incorporation.

BHK cells were found to incorporate approximately 40-50% of the total labeled acyl-CoA at concentration of 5 μM into the whole cells in

each individual experiment over an incubation period of 20 mins. The percentage of incorporated acyl-CoA is in agreement with that found by Fischer et al., (1967) and Curtis et al., (1975b). 21% of the total incorporated is present in the plasma membrane. This clearly reflect a remarkably rapid incorporation of the acyl-CoA used which in turn reflect a fast metabolism of the lipid molecules. Previous work have shown that exogenously supplied fatty acids are incorporated and alter the fatty acid composition of membrane phosphatides (Horwitz et al., 1974).

The percentage of fatty acyl-CoA incorporation was reduced however, in presence of serum. A 43% reduction in the uptake was seen under serum conditions in comparison with the experiment carried out in serum-free medium. As mentioned earlier, this decrease in the uptake might be due to the presence of lipids or an acyl-binding substance.

The results presented in the first section of this thesis are partly in agreement and in conflict (see Table.29) with other worker's results.

The differences in results could be due to many factors (Table.30), among which are, cell type in the case of the work presented by Curtis and his co-workers, (1975), the method used to measure cell substratum adhesion and the media in which experiments are performed concerning the data by Hoover et al., (1977) and Schaeffer and Curtis (1977).

Table. 29. A summary comparing the effect of acyl-CoAs used in this work, with the effect of fatty acid (not bound to CoA) used by others.

Fatty acid	Effect on Attachment(†)			
	Present work (As acyl-CoAs)	Curtis et al.,1975b.c	Hoover et al.,1977	Schaeffer & Curtis, 1977
Stearic	+/-	+	≥/-	+
Oleic	≥/-	+	=	ND
*Linoleic	≥	+	-	-
Linolenic	=	-	-	-
*Arachidonic	=	-	-	ND

+/-: Increase then decrease at high concentrations

- Decrease; + Increase

≥: Level with control or slightly higher

=: Level with the control

ND: Not done.

* Both were found to reduce adhesion in my work when used in the free form.

† Cell-cell or cell-substrate adhesion.

Table.30. Comparison of cell type and adhesion conditions of the present work with others.

Workers	Cell type	medium	adhesion assay
Curtis, et al., 1975b&c	Neural cells of the retina	Hanks + 199-CM-free medium	Cell-cell
Hoover, et al. 1977	BHK, CHO	HBS solution + 15 mM Hepes	Cell-cell and cell-polystyrene
Schaeffer and Curtis, 1977.	Mouse L929	Hanks	Cell-polystyrene
Present work	BHK	3% serum or serum-free Ham's F-10	Cell-polystyrene

It should be noted also that in my experiments the acyl-form rather than acid form was used, which might have led to a different mechanism of action. A general summary is given bellow illustrating the main effects of the acyl-CoAs on BHK cell adhesion, spreading and viability under serum-free conditions.

Table.31.A summary of the effects of acyl-CoAs on BHK cell functions.

	Adhesion		Spreading	Viability
	1.75-7	14-28μM		
Stearoyl-CoA	+	-	-	Poor (>7μM)
Oleoyl-CoA	≥	-	-	Poor (14μM & >)
Linoleoyl-CoA	≥	=	=	Normal
Linolenoyl-CoA	=	=	=	Normal
Arachidonoyl-CoA	=	=	=	Normal

\+ Increase; - Decrease

= Level with control

≥ Level or slightly higher than the control.

Section 4.2. **Cell activation and adhesion.**

Because the effects of some fatty acids could be explained in terms of activation as in leukocytes, I looked at some of the systems known to activate adhesion in leukocytes to see if there is evidence for them in BHK cells.

Activation of cell functions, in particular cell adhesion is a very well established phenomena for myeloid and other cell types (Hoover et al., 1984; Weissmann et al., 1986; Nagao et al., 1989). As mentioned earlier in the introduction, blood cells can be activated to adhere to different substrata. Many factors have been implicated to be responsible for this activation. These factors include in particular the product of phospholipid metabolism. Diacylglycerol, inositol trisphosphate arachidonic acid metabolites, phorbol esters and calcium ionophores are a very well known modulator of cell adhesion of many cell types (see Berridge, (1987) for review).

The main findings in this section are as follow:

1. Phospholipase A_2 inhibitors, bromophenacyl bromide and mepacrine inhibited BHK cell adhesion to polystyrene tissue culture dishes. A reduction of approximately 20% of the control value was found with mepacrine at a concentration of 12.5 μM. Bromophenacyl bromide however, reduced the adhesion in a dose dependent manner, the higher the concentration is the greater the effect. A 98% inhibition was seen with 8μM BPB in serum-containing medium. The inhibitory effect was less pronounced in serum-free conditions. Cell adhesion was irreversibly inhibited. Cells washed and resuspended in fresh medium after 20 mins incubation with BPB were unable to adhere even for a long period of incubation in tissue culture dishes.

2. Arachidonic acid metabolites. Leukotriene B_4 (LTB_4) at 0.125 and 1.25 μM was found to increase BHK and B10D2 cell-cell adhesion compared with untreated cells. Prostaglandin E_1 (1-50μM) also increased BHK cell adhesion to polystyrene tissue culture dishes. Prostacyclin however kept the adhesion at control level (see later).
3. The calcium mobilizer inositol trisphosphate and the inhibitor of diacylglycerol kinase, were both found to decrease BHK as well as B10D2 cell adhesion. A small but significant decrease was seen with IP_3 at 20 μM and higher concentrations. In contrast the adhesion was sharply reduced in presence of diacylglycerol kinase inhibitor at 18μg/ml and higher concentrations.

4. No synergistic effect between PMA and ionomycin was found on BHK cell adhesion. PMA (0.5-4μg/ml) added alone increased BHK cell adhesion. Ionomycin at 8-16μM concentrations also in very low calcium medium slightly enhanced the adhesion.

PMA in combination with PGE_1 did not affect BHK cell adhesion. The spreading area however, was increased. The addition of PGE_1 by itself enhanced BHK cell adhesion.

In a comparison experiment, PMN leukocyte adhesion was found to increase significantly when stimulated with PMA. PGE_1 on the other hand decreased PMN adhesion stimulated with PMA but no effect was seen on the adhesion of unstimulated (base line) PMN leukocytes.

PMA, Ionomycin, PGE_1, were found to affect the BHK cell shape. The cells appear in elongated shape when treated with PMA or ionomycin (Plate.8).

Over an incubation period of 65 mins, PGE_1 treated cells show a process like growth and flattening. Pseudopodal projections were seen all over the cell surface in comparison with untreated cells

which still in fibroblastic appearance (Plate.9).

---

1. Phospholipase A_2 inhibitors.

The mechanism by which bromophenacyl-bromide and mepacrine reduced BHK cell adhesion might be related to the inhibition of PLA_2.

Bromophenacyl-bromide was shown to inhibit PLA_2 activity irreversibly in cell-free system (Roberts et al., 1977).

Johns and Peach (1987) have demonstrated that BPB pretreatment of endothelial cells blocked phospholipase activity as measured by attenuation of the release of ^3H-arachidonate from prelabeled endothelial cells. It was shown also that BPB suppressed the production of superoxide anion and inhibited lysosomal enzyme secretion in fMLP stimulated neutrophils (Takenawa et al., 1986).

Mepacrine on the other hand was found to block aggregation, secretion, phosphorylation of 47 Kd protein and myosin light chain, lysophosphatidic acid formation, the release of arachidonic acid and the formation of thromboxane B_2 in platelets induced with thrombin (McCrea et al., 1985)

Evans and Lanham,(1986) also demonstrated that mepacrine impaired the aggregation of SV40-3T3 cells. The inhibitory effect was greater when the monolayer was treated with the drug for two hours prior to the adhesion assay. They showed also that up to 50 μM concentration did not affect the viability. Mepacrine was found to block both chemotaxis and the release of arachidonic acid in rabbit leukocytes (Hirata, et al., 1979).

Therefore, the biological activities of mepacrine and BPB may be explained by a direct or indirect inhibition of PLA_2. The effect however, may be the consequence of a non-specific generalized action due to the acylation of molecules other than PLA_2. Nevertheless,

the demonstration that the acylation of the histidine residue close to the active site by BPB is specific (Volwerk, et al., 1974), the effect is probably due to an action on PLA_2 and not other molecules. In contrast, mepacrine could have acted indirectly on PLA_2. It might have some how reduced the availability of calcium needed for calcium-dependent activation of PLA_2 or due to an action on the substrate rather than the enzyme itself. In this respect, it was demonstrated that mepacrine was without effect on phospholipids hydrolysis, arachidonic acid release and prostaglandin synthesis in platelets activated by calcium ionophore A23187 (Vallee, et al., 1979).

Therefore, whatever the mechanism of action on PLA_2 activity, the effect could be due in general to the inhibition of arachidonic acid release. A number of arachidonic acid metabolites have been shown to increase cell adhesion. Thus the inhibition of the synthesis of such metabolites might be the cause of the observed effect on cell adhesion.

The inability of mepacrine to reduce cell adhesion in similar manner to that observed with bromophenacyl bromide could be due to a partial inhibition of PLA_2 activity unlike bromophenacyl bromide.

It is still too early to explain in satisfactory fashion the effects of mepacrine and bromophenacyl-bromide on BHK cell adhesion. It is worth looking in a further work to the activity of PLA_2, arachidonic acid release and prostaglandin synthesis from BHK cells treated with the above agents.

2. Arachidonic acid metabolites.

Arachidonic acid metabolites are known to have multiple functions. They are involved in the regulation of immune responses, adhesion of platelets and leukocytes, cytotoxicity and phagocytosis (Higgs et

al., 1979). The wide spread functions that prostaglandins exhibit made them a very important molecules to be studied. Prostaglandins have been shown to act via a membrane receptor coupled to adenylate cyclase (Schafer et al.,1979). The activation of this enzyme leads to the accumulation of cAMP which serves as an intracellular second messenger that may affect many cellular functions.

Among the prostaglandins studied is prostacyclin (PGI_2), prostaglandin E_2 and prostaglandin E_1.

Prostacyclin failed to influence BHK cell adhesion using concentration up to 5 μg/ml. PGI_2 and PGE_2 were found however to increase BHK cell spreading area in 3% serum medium but they did not affect cellular adhesion under the same conditions. The inability of PGI_2 and PGE_2 to alter cell adhesion is unclear. It might be suggested however, that their effectiveness in increasing intracellular cAMP was minimal, and therefore, they were not sufficient enough to alter the adhesion mechanisms.

Prostaglandin E_1 on the other hand was found to enhance BHK cell adhesion, spreading and morphological change in cell shape. Prostaglandin E_1 effect, like other prostaglandins, could be mediated by an increase in intracellular cAMP.

In cultured fibroblasts, the activity of PGE_1 in stimulating the production of cAMP was found to be more than that of PGI_2 which might be due to the instability of PGI_2 (Murota et al., 1979). This may explain the effect seen with PGE_1 unlike PGI_2.and PGE_2 which did not influence BHK cell adhesion.

Prostaglandin E_1 was shown to enhance the rate of initiation of DNA replication in Swiss 3T3 cells simulated by $PGF_{2\alpha}$ alone or with insulin (Otto et al., 1982). It was also found to inhibit PMN leukocyte adhesion to glass in plasma (Chuang, et al., 1980) and inhibited PMA or fMLP stimulated PMN adherence to plastic or

endothelial cells in a dose dependent manner (Chopra & Webster 1988). These effects were linked to an increase in the content of cellular cAMP which serves as intracellular second messenger in initiating many processes that lead to the final response.

In contrast, Pearson et al., (1979), Showed that the addition of dibutyryl-cAMP or PGE_1, significantly increased granulocyte adhesion to endothelial cells. They suggest that increasing granulocyte cAMP potentiated granulocyte adhesion to endothelial cells. It is also known that treatment of fibroblasts with dibutyryl-cAMP leads to an increase in overall adhesiveness to the substratum (Johnson et al 1972). This is based on the fact that this treatment decreases the rate at which trypsin or EGTA removes these cells from the substratum.

Therefore, PGE_1 enhancement of BHK cell adhesion could have been mediated by an increment in intracellular cAMP content. The mechanism linking the increase in cAMP and enhancement of cell adhesion is unclear. However, cAMP could have activated cAMP dependent kinase which might have in turn phosphorylated some target substrate proteins. The phosphorylation of such proteins could have activated a process(es) which is linked to a mechanism controlling cell adhesion.

The effect might be due also to the increase in cell contact area with tissue culture dishes. This might have made the cells firmly attached and thus more resistant to the detachment force during the washing process. Previous studies however, have indicated that the amount of surface contact between cells and substratum alone is not a major consideration in adhesion, other factors such ionic forces, secreted granular constituent and cell surface proteins must constitute the major determination of adherence (Webster et al., 1986).

Changes in cellular morphology have been reported to occur in many biological events including hormonal stimulation and in many instance, cyclic AMP has been implicated as the second messenger. For example, rat mesangial cells have been reported to undergo a change in cell shape following hormonal stimulation (Kreisberg et al., 1984). This alteration in cellular morphology occurs minutes after maximum increase in intracellular cyclic AMP and can be mimicked by dibutyryl-cAMP.

The neuronal shape, the increase in the number of narrow cytoplasmic projections and flattening exhibited by BHK cells in response to PGE_1, could be therefore explained in terms of an increase in intracellular cyclic AMP. It is not certain however, how cAMP initiated this events. Hsie and Puck, (1971), described a reversible transformation in structure of chinese hamster cells by dibutyryl cyclic AMP and testosterone, and presented evidence that this metamorphosis involves action of cellular microtubules. Cyclic AMP was also found to restore morphological characteristics of normal fibroblasts in sarcoma cells (Johnson et al., 1971). Treatment of 3T3 and L929 fibroblastic cells with dibutyryl-cAMP was found to change their shape accompanied by altered intracellular distribution of microfilaments and microtubules (Willingham & Pastan, 1975).

Therefore, the effect of PGE_1 on BHK cell shape could be due to a rearrangement in the distribution of microtubules and microfilaments as a result of a presume increase in intracellular cyclic AMP.

LTB_4 another arachidonic acid metabolite increased significantly BHK and slightly B10D2 endothelial cell-cell adhesion. The mechanism of action could be linked to the ability of LTB_4 to increase intracellular calcium which might have led directly or in indirect fashion to the observed enhancement of cell-cell adhesion.

It was suggested that LTB_4 can acts as calcium ionophore (Volpi et al., 1984) and hence might have affected the adhesion in this manner.

LTB_4 was also found to bind specifically to human neutrophils and produced a significant rise in intracellular calcium (Yamazaki et al., 1989) and have been shown to augment neutrophil-endothelial adhesion (Hoover et al., 1984).

3. Diacylglycerol kinase inhibitor and IP_3.

The diacylglycerol kinase inhibitor (6-[2-[4-[(4-fluorophenyl) phenylmethelene]-1-piperidinyl]-7-methyl-5H-thiazolo-[3,2-a] pyrimidine -5-one), was found to inhibit diacylglycerol kinase (the enzyme that converts diacylglycerol to phosphatidic acid) in human red blood cell membrane and in intact platelets (de Chaffoy de Courcelles, et al., 1985). It has been shown also, to potentate the secretion and aggregation responses in thrombin stimulated platelets (Nunn & Watson 1987), and enhance the superoxide generation from stimulated neutrophils (Muid et al., 1987).

The exact mechanism underlying the reduced cell adhesion with diacylglycerol kinase inhibitor is unclear. Diacylglycerol is known to be the cellular activator of protein kinase C (Nishizuka, 1984). Therefore, the activation of protein kinase C will lead to the phosphorylation of specific target proteins which are thought to contribute to the final response. On these bases the above effects might be mediated by the activation of protein kinase C as a result of the accumulation of diacylglycerol. Another possible mechanism is the hydrolysis of diacylglycerol by diacylglycerol lipase to form monoacylglycerol which is further hydrolyzed to release arachidonic acid (Berridge, 1987). Since the later is the precursor of eicosanoids, it might have been metabolized to release PGs and LTs.

The accumulation of such biologically active agents may have somehow caused the observed effect on cell adhesion. Nevertheless, other effects such as nonspecific action of the drug on the chemical and the physical properties of cell membranes and cellular compartments, cannot be excluded. It is clear further biochemical investigations are required.

The small decrease in BHK and B10D2 endothelial cell adhesion caused by quite high concentrations of IP_3 could be due to an increase in intracellular calcium from an internal or external source. It was shown that external IP_3 could induce in intact cells an influx of external calcium through transmembrane channels (Michell, 1975; Kuno and Gardner, 1987). IP_3 was found also to release calcium from intracellular stores in digitonin-permeabilized human neutrophils (Prentki, et al., 1984). Cells incubated with tritiated IP_3 were also found to bind approximately $27x10^3$ molecule/cell (0.3% of total) after 10 mins incubation at 37°C. Thus, after trypsinization, it is presumable that cells were permeabilized and therefore IP_3 could have acted intracellularly to release calcium from intracellular stores. The peripheries of many cell types were already shown to be altered by trypsin treatment during their dissociation (Barnard, et al., 1969; Poste, 1971).

This in fact could be the case, since cells which are allowed to recover from trypsin effect for 60 mins at 37°C, were mostly unresponsive to IP_3 concentrations which reduced the adhesion of cells which were not preincubation before cell attachment assay. The mechanism underlying a presume increase in intracellular calcium and the observed decrease in cell attachment is unclear.

On the other hand, it is known that IP_3 is a highly charged

molecule to which cells are normally nonpermeable (Europe-Finner and Newell, 1985). Therefore, IP_3 could have interacted non-specifically with the plasma membrane and therefore might have affected the total net negative charge and thus electrostatic forces which in turn might have affected cell adhesion. As a charged molecule, IP_3 could have chelated divalent cations from the adhesion medium which led to a reduction in cell adhesion.

4. Phorbol myristate acetate and ionomycin.

The tumour promoter phorbol esters are a class of low molecular weight nonpeptide mitogens active in many systems, including keratinocytes, human fibroblasts and Swiss 3T3 cells (Diamond et al., 1980).

Phorbol myristate acetate (PMA) was found to increase neutrophil adherence to plastic tissue culture (Webster et al., 1986), and endothelial cells (Chopra and Webster, 1988), and enhanced PMN spreading on glass or plastic (Hoover et al., 1987). Neutrophils stimulated with PMA revealed also extensive spreading and numerous filamentous process that extend from the cell (Webster et al., 1986). Nagao et al., (1989), presented a data showing a correlation between an increase in C kinase activity and cell-cell contacts formation associated with rearrangement of keratin intermediate filaments in human carcinoma cell line.

PMA action on cellular function is usually linked to its ability to bypass the signal transduction pathway and the direct activation of protein kinase C which in turn phosphorylates specific target proteins (Kikkawa and Nishizuka, 1986).

The increase of BHK cell adhesion when treated with PMA could be therefore the result of the activation of protein kinase. The mechanism by which activated protein kinase increased cell adhesion

is far from clear. The effect could be due to an increase in the expression of a molecule that is important in adhesion or phosphorylation of target proteins that are directly or indirectly related to cell adhesion. In this respect it was shown (Chopra and Webster, 1988) that PMA increased the expression of a surface glycoprotein (Mol) in neutrophil, previously shown to play an important role in PMN leukocytes adhesion (Harlan et al., 1985).

Protein kinase C phosphorylation of target proteins has been also shown as a mechanism for modulating cell adhesion (Danilov and Juliano, 1989; Shaw et al., 1990)

The effect of PMA on cell spreading and morphological change could be linked to an action on microfilament network. It was previously shown that PMA-stimulated neutrophils, spread widely on the endothelial monolayer or on plastic, whereas, neutrophils treated with cytochalasin B before PMA addition, rounded up and failed to spread (Webster et al., (1986). Shaw et al., (1990), also demonstrated that PMA treated macrophages resulted in a marked alteration of cell shape. Using rhodamine-phalloidin staining they showed that the pattern of actin changed after PMA activation, and they also noted an increase in the number of bundles of actin filaments. In this respect, phorbol esters were found to induce actin polymerization in human leukocytes (Rao, 1985). The effects of PMA on cell morphology are not however, the result of an overall generalized toxicity, since in low-density cultures of some cells such as chick embryo fibroblasts, the growth rate in PMA-containing medium may be not different from control medium, though in other cell types (e.g., 3T3 mouse fibroblast), it may cause an inhibition of cell division for up to 48 hours after which cells regain their normal growth (Diamond et al.,1980, for review). The long duration of PMA effect on cell shape could be due to the

phosphate attached to the substrate proteins of protein kinase C. It was indicated that such phosphates are frequently resistant to the action of phosphatases and therefore the consequence of these phosphorylation reaction may persist for a long period (Kikkawa and Nishizuka, 1986).

Ionomycin did not affect in clear manner cell adhesion, though, a small increase was seen when high concentrations were used and the adhesion assay was performed in calcium-free medium.

Ionomycin which did not affect cell adhesion in 3% serum-containing medium when added alone or with PMA, was found however, to cause a reversible morphological change mostly similar to that caused by PMA. The effect persist till the medium is changed. This could be explained in terms of the continuous influx of calcium which is known to affect many cellular functions.

BHK cell adhesion was kept at the control level when low concentration of PMA was combined with PGE_1. The lack of effect of PGE_1 on PMA stimulated BHK cells is unclear. Since PGE_1 increased BHK cell adhesion when added alone to the cells, it could be argued therefore that PMA might have antagonised the effect of PGE_1 leading to an adhesion leveled with untreated cells.

In a comparison experiment, PMA increased neutrophil adhesion extensively to polystyrene tissue culture dishes. PGE_1 on the other hand at 10 μM concentration reduced neutrophil adhesion significantly ($P<0.001$) when stimulated with PMA. The adhesion in the absence of PMA however, was not affected by PGE_1. This might be in agreement with previous work showing that PGE_1 did not affect PMN base-line adherence to nylon (McGillen et al., 1980).

The inhibitory effect caused by PGE_1 on PMA stimulated neutrophil adhesion could be mediated by an increase in intracellular cAMP since it was demonstrated that both PGE_1 and

cAMP inhibited granulocyte adhesion to glass capillary tubes (Bryant and Sutcliffe, 1974).

Generally, BHK fibroblast cell adhesion, spreading and cell morphology were found to be modified by a number of compounds known to activate many cellular functions in other cell types in particular blood cells. Therefore, BHK cell functions could be regulated by activation events, a phenomenon known for other cell types. A summary judging this hypothesis is given below.

Table.32. A summary judging the hypothesis that BHK fibroblasts might have activation event like leukocytes.

Agent	Attachment*		Spreading		morphology	
	PMN	BHK	PMN	BHK	PMN	BHK
LTB_4	+	+	NK	NK	NK	NK
$[Ca^{++}]_i$ (Ionophore)	+	±	+	-	NK	+
$[Ca^{++}]_i$ (IP_3)	Nk	+	NK	NK	NK	NK
PMA	+	+	+	+	+	+
PLA_2 inhibitors	+	+	NK	+	NK	+
PGs	+	±	NK	+	NK	+

\+ Positive response (increase or decrease)
\- Negative response (no effect)
± No clear response
NK Not known
* Cell-cell or cell-substratum adhesion.

4.3. Concluding remarks.

In conclusion BHK cell adhesion, spreading and morphology were found to be modulated by fatty acids and acyl-CoAs, arachidonic acid metabolites, calcium mobilizers, phospholipase inhibitors and protein kinase C activators. Although a number of the agents used did not affect the above cellular function to a big extent, it is apparent that there are many responses. The lack or small effect found with some of these factors could be investigated in future work.

The mechanism underlying these effects could be explained on one hand as a result of an alteration in membrane properties, in particular membrane fluidity due to the substitution of fatty acids content of phospholipids. On the other hand, the modulation of cell functions by the other factors (calcium/protein kinase C modulators), could be a consequence of general activation of intracellular events that are translated into cellular functions. It is still too early however, to speculate with certainty that the effects are mediated by activation events and not the result of other effects. Thus, a big deal of work is still to be done in the future to clarify these matters.

4.4. Future directions.

1. Technical.

It is worth improving the procedure of washing the cells after the adhesion assay, in a way that removing non-adherent cells does not affect attached ones, though an experiment carried out in this purpose did not show a real effect of the washing procedure on already attached cells.

2. Experimental.

i. Acyl-CoAs. It will be of interest to test the effect of other

acyl-CoAs, to use albumin-depleted and/or delipidated serum medium, to measure membrane fluidity, and identification of the nature of fatty acids in membrane phospholipids and in which position the substitution of the fatty acids took place.

ii. Concerning calcium/protein kinase C pathway modulators it is worth testing their effects on cell adhesion and spreading in different media such as calcium-free medium and to use other concentrations in addition to those used in the present work. Another important piece of research is to look at protein kinase C activity and its phosphorylated target proteins when agents such as phorbol esters or calcium mobilizers are used.

Chapter V

References

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J.D. (1983). Molecular biology of the cell. Garland Publishing, Inc. New York & London.

Allen, J.M. & Cook, G.M.W. (1970). A study of the attachment phase of phagocytosis by murine macrophages. *Expl. Cell Res.* **59**: 101-116.

Ambrose, E.J., James, A.M. & Lowick, J.H.B. (1956). Differences between the electrical charge carried by normal and homologous tumour cells. *Nature (London)*, **177**: 576-577.

Arnaout, M.A., Hakim, R.M., Tood III, R.F., Dana, N., Colten, H.R. (1985). Increased expression of an adhesion-promoting surface glycoprotein in the granulocytopenia of hemodialysis. *N. Eng. J. Med.* **312**: 457-462

Atherton, A. & Born, G.V.R. (1972). Quantitative investigations of the adhesiveness of circulating polymorphonuclear leukocytes to blood vessel walls. *J. Physiol.* **222**: 447-474.

Baenziger, N.L., Brodie, G.N. & Magerus, P.W. (1971). A thrombin-sensitive protein of human platelet membranes. *Proc. Natl. Acad. Sci. (USA)*. **68**: 240-243.

Baggiolini, M., Schnyder, J. & Bretz, U. (1979). Lysosomal enzymes and neutral proteinases as mediators of inflammation. *In, advances in inflammation research,* Weissmann, G. Samuelsson, B., Paoletti, R. Eds. **Vol. 1**: 263-272. Raven Press, New York.

Baker, T.S., Caspar, D.L.D., Hollinghead, C.J. & Goodenough, D.A. (1983). Gap junction structures. IV. Asymmetric features revealed by low-irradiation microscopy. *J. Cell Biol.* **96**: 204-216.

Ballou, L.R. & Cheung, W.Y. (1983). Marked increase of human platelet phospholipase A_2 activity *in vitro* and demonstration of an endogenous inhibitor. *Proc. Natl. Acad. Sci. (USA)*. **80**: 5203-5207.

Ballou, L.R. & Cheung, W.Y. (1985). Inhibition of human platelet PLA_2 activity by unsaturated fatty acids. *Proc. Natl. Acad. Sci. (USA).* **82:** 371-375.

Bangham, A.D., De Gier, J. & Greville, G.D. (1967). Osmotic properties and water permeability of phospholipid liquid crystals. *Chem. Phys. Lipids*, **1:** 225-246.

Barnard, P.J., Weiss, L. & Ratcliffe, T. (1969). Changes in the surface properties of embryonic chick neural retina cells after dissociation. *Expl. Cell Res.* **54:** 293-301.

Barsky, S.H., Rao, C.N., Williams, J.E. & Liotta, L.A. (1984). Laminin molecular domains which alter metastasis in murine model. *J. Clin. Invest.* **74:** 843-848.

Baumgartner, H.R., Tranzer, J.P. & Studer, A. (1967). An electron microscopic study of platelet thrombus formation in the rabbit with particular regard to 5-hydroxytryptamine release. *Thrombosis et Diathesis Haemorrhagica*. **18:** 592-604.

Beaudry, G.A., King, L., Daniel, L.W. & Waite, M. (1982). Stimulation of deacylation in madin-Darby canine kidney cells. Specificity of deacylation and prostaglandin production in 12-O-tetradecanoyl-phorbol-13 acetate-treated cells. *J. Biol. Chem.* **257:** 10973-10977.

Bell, R.L. & Majerus, P.W. (1980). Thrombin-induced hydrolysis of phosphatidyl-inositol in human platelets. *J. Biol. Chem.* **255:** 1790-1792.

Berger, M., O´Shea, J., Cross, A.S., Folks, T.M., Chused, T.M., Brown, E.J & Frank, M.M. (1984). Human neutrophils increase expression of C3 bi as well as C3b receptors upon activation. *J. Clin. Invest.* **74:** 1566-1571.

Berridge, M.J. (1984). Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:** 345-360.

Berridge, M.J.(1986). Inositol trisphosphate and calcium mobilization. *In calcium and the cell.* Ciba. Fond. Symp. John Wiley & Sons Ltd. UK. **122: 39-49**

Berridge, M.J. (1987). Inositol triphosphate and diacylglycerol: Two interacting second messengers. *Ann. Rev. Biochem.* **56**: 159-193.

Berridge, M.J. & Irvine, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature, (London)*, **312**: 315-321.

Besterman, J.M., Pollenz, R.S., Booker, E.L. & Cuatrecasas, P. (1986). Diacylglycerol-induced translocation of diacylglycerol kinase: use of affinity-purified enzyme in reconstitution system. *Proc. Natl. Acad. Sci. (USA)*. **83**: 9378-9382.

Beug, H., Gerisch, G., Kempff, S., Riedel, V. & Gremer, G. (1970). Specific inhibition of cell contact formation in *Dictyostelium* by univalent antibodies. *Expl. Cell Res.* **63**: 147-158.

Beug, H., Katz, F.E. & Gerisch, G. (1973). Dynamics of antigenic membrane sites relating to cell aggregation in *Dictyostelium discoideum. J. Cell Biol.* **56**: 647-658.

Bierman, A.(1955).Electrostatic forces between non-identical colloidal particles. *J. Cell. Sci.* **10**: 231-245.

Bockenstedt, L.K. & Goetzl, E.J. (1980). Constituents of human neutrophils that mediate enhanced adherence to surfaces. Purification and identification as acidic proteins of the specific granules. *J. Clin. Invest.* **65**: 1372-1381.

Bouziges, F., Simon-Assman, P., Leberquier, L. Haffen, K.& Kedinger, M (1989). Glycosaminoglycan expression in intestinal epithelial skin-fibroblastic cell cocultures. Fibroblastic cell-mediated effects of glucocorticoids. *J. Cell sci.* **92**: 679-685.

Brackenbury, R., Thiery, J.P., Rutishauser, U. & Edelman, G.M. (1977). Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. *J. Biol. Chem.* **252**: 6835-6840.

Bray, R., Abrams, S.& Brahmi, Z. (1983). Studies on the mechanism of human natural killer cell-mediated cytolysis. I. modulation by dexamethasone and arachidonic acid. *Cell Immunol.* **78**: 100-113.

Brooks, D.E., Millar, J.S., Seaman, G.V.F. & Vassar, P.S. (1967). Some physicochemical factors relevant to cellular interactions. *J. Cell Physiol.* **69**: 155-168.

Bruno, J.J.,Taylor, L.A.& Droller, M.J.(1974). Effect of prostaglandin E_2 on human platelet adenyl cyclase and aggregation. *Nature, London.* **251**: 721-723.

Bruynzeel, P.L.B., Kok, P.T.M., Hamelink, M.L., Kijne, A.M.& Verhagen, J. (1985). Exclusive leukotriene C_4 synthesis by purified human eosinophils induced by opsonized zymosan. *FEBS. Lett.* **189**: 350-354.

Bryant, R.E. & Sutcliffe, M.C. (1974). The effect of 3′,5′-adenosine mono-phosphate on granulocyte adhesion. *J. Clin. Invest.*. **54**: 1241-1244.

Butcher, E.C., Scollay, R.G.& Weissman, I.L.(1979). Lymphocyte adherence to high endothelial venules: Characterization of a modified *in vitro* assay, and examination of the binding of syngeneic and allogeneic lymphocyte populations. *J. Immunol.* **123**: 1996-2003.

Cazenave, J.P., Dejana, E., Kinlough-Rathbone, R.L., Richardson, M., Packham, M.A.& Mustard, J.F. (1979). Prostaglandin I_2 and E_1 reduce rabbit and human platelet adherence without inhibiting serotonin release from adherent platelets. *Thromb. Res.* **15**: 273-280.

Chadwick, C.C, Saito, A. & Fleischer, S. (1990). Isolation and characterization of the IP_3 receptor from smooth muscle. *Proc. Natl. Acad. Sci. (USA).* **87**: 2132-2136.

Chapman, D. (1973). Some recent studies of lipid, lipid cholesterol and membrane systems. *In biological membranes*, vol. **2**, Chapman, D. and Wallach, D.F.H. Eds. Acadimic Press, London, New York. pp. 91-144.

Charo, I.F., Yuen, C.& Goldstein, I.M. (1985). Adherence of human polymorphonuclear leukocytes to endothelial monolayers: Effect of temperature, divalent cations, and chemotactic factors on the strength of adherence measured with a new centrifugation assay. *Blood.* **65**: 473-479.

Charo, I.F., Yuen, C., Perez, H.D.& Goldstein, I.M. (1986). Chemotactic peptides modulate adherence of human polymorphonuclear leukocytes to monolayers of cultured endothelial cells. *J. Immunol.* **136**: 3412-3419.

Cheresh, D.A., Harper, J.R., Schulz, G.& Reisfeld, R.A. (1984). Localization of the gangliosides GD_2 and GD_3 in adhesion plaques and on the surface of human melanoma cells. *Proc. Natl. Acad. Sci. (USA).* **81**: 5767-5771.

Cheresh, D.A., Pierschbacher, M.D., Herzig, M.A.& Mujoo, K. (1986). Dicsalogangliosides GD_2 and GD_3 are involved in the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. *J. Cell Biol.* **102**: 688-696.

Chopra, J.& Webster, R.O. (1988). PGE_1 inhibits neutrophil adherence and neutrophil-mediated injury to cultured endothelial cells. *Am. Rev. Respir. Dis.* **138**: 915-920.

Chuang, H.Y.K., Mohammad, S.F.& Mason, R.G. (1980). Prostacyclin (PGI_2) inhibits the enhancement of granulocyte adhesion to cuprophane induced by immunoglobulin G. *Throm. Res.* **19**: 1-9.

Collins, M. (1966). Electrokinitic properties of dissociated chick embryo cells. I and II. *J. Expl. Zool.* **163**: 23-47.

Coman, D.R. (1944). Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. *Cancer Res.* **4**: 625-629.

Conti,P., Cifone, M.G., Alesse, E., Reale,M., Cancelli, A., Angeletti, P.U.& Dinarello, C.A. (1987). Effect of polyclonal antibody against interleukin 1 on natural killer cell activity. *Advances in the biosciences*, **66**: 501-509.

Conti,P., Cifone, M.G., Alesse, E., Reale, M., Fieschi, C., Fiore, S., Bologna, M.& Dinarello, C.A. (1986). Synergistic activation of natural killer activity by human recombinant interferon alpha or gamma and indomethacin. *Int.J. Immunotherapy*, **11 (4)**: 289-293.

Cook, G.M.W., Heard, D.H. & Seaman , G.V.F. (1961). Sialic acids and the electrokinitic charge of the human erythrocyte. *Nature, (London)*, **191**: 44-47.

Cowin, p.,Mattey, D.& Garrod, D.R.(1984). Identification of desmosomal surface components (desmocollins) and inhibition of desmosome formation by specific Fab´. *J. Cell Sci.* **70**: 41-60.

Cox, C.C. Dougherty, R.W., Ganong, B.R., Bell, R.M., Niedel, J.E.& Snyderman, R. (1986). Differential stimulation of the respiratory burst and lysosomal enzyme secretion in human polymorphonuclear leukocytes by synthetic diacylglycerols. *J. Immunol.* **136**: 4611-4616.

Culp, L.P., Laterra, J., Lark, M.W., Beyth, R.J.& Tobey, S.L. (1986). Heparan sulphate proteoglycan as mediator of some adhesive responses and cytoskeletal reorganization of cells of fibronectin matrices independent versus cooperative functions. *Ciba. Found. Symp.* **124**: 158-183.

Cunningham, B.A., Hoffman, S., Rutishauser, U., Hemperly, J.J.& edelman, G.M. (1983). Molecular topography of the neural cell adhesion molecule N-CAM: Surface orientation and location of sialic acid-rich and binding regions. *Proc. Natl. Acad. Sci. (USA)*. **80**: 3116-3120.

Cunningham, B.A., Leutzinger, Y., Gallin, W.J., Sorkin, B.C.& Edelman, G.M. (1984). Linear organization of the liver cell adhesion molecule L-CAM. *Proc. Natl. Acad. Sci. (USA)*. **81**: 5787-5791.

Curtis, A.S.G. (1960). Cell contacts: Some physical considerations. *American Naturalist*. **94**: 37-56.

Curtis, A.S.G. (1962). Cell contact and adhesion. *Biol. Rev.* **37**: 82-129.

Curtis, A.S.G. (1964). The adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell Biol* . **19**: 199-215.

Curtis, A.S.G. (1967). The cell surface: Its molecular role in morphogenesis. Logos Press, London: Academic Press, London.

Curtis, A.S.G. (1969). The measurement of cell adhesion by an absolute method. *J. Embryol. Expl. Morphol.* **22**: 305-325.

Curtis, A.S.G. (1972). Intra- and inter-membrane interactions of the cell surface. *Subcell. Biochem.* **1**: 179-196.

Curtis, A.S.G. (1973). Cell adhesion. *Prog. Biophys. Mol. Biol.* **27**: 315-386.

Curtis, A.S.G. (1987). Cell activation and adhesion. *J.Cell Sci.* **87**: 609-611.

Curtis , A.S.G., and Forrester, J.V. (1984). The competitive effects of serum proteins on cell adhesion. *J. Cell Sci.* **71**: 17-35.

Curtis, A.S.G.& McMurray, H.(1986). Conditions for fibroblast adhesion without fibronectin. *J. Cell Sci.* **86**: 25-33.

Curtis, A.S.G., Campbell, J. & Shaw, F.M. (1975a). Cell surface lipids and adhesion. The effect of lysophosphatidyl compounds, phospholipase A_2 and aggregation- inhibiting protein. *J. Cell Sci.* **18**: 347-356.

Curtis, A.S.G., Chandler, C. & Picton, N.(1975c). Cell surface lipids and adhesion. The effects on cell adhesion of changes in plasmalemmal lipids. *J. Cell Sci.* **18**: 375-384.

Curtis, A.S.G., Forrester, J.V.& Clark, P. (1986). Substrate hydroxylation and cell adhesion. *J. Cell Sci.* **86**: 9-24

Curtis, A.S.G., Forrester, J.V., McInnes, C.& Lawrie, F. (1983). Adhesion of cell to polystyrene surfaces. *J. Cell Biol.* **97**: 1500-1506.

Curtis, A.S.G., Shaw, F.M. & Spires, V.M.C. (1975b). Cell surface lipids and adhesion. The turnover of lipid components of the plasma-lemma in relation to cell adhesion. *J. Cell Sci.* **18**: 357- 373.

Dan, K. (1936). Electrokinetic studies of marine ova. III, Physiol. Zool. **9**: 43-57.

Danilov, Y.N & Juliano, R.L. 1989. Phorbol ester modulation of integrin-mediated cell adhesion: A postreceptor event. *J. Cell Biol.* **108**: 1925-1933.

Dawson, A.P. (1985). GTP enhances IP_3-stimulated calcium release from rat liver microsomes. *FEBS. Lett.* **185**: 147-150.

de Chaffoy de Courcelles, D., Roevens,.P. & Van Belle, H., (1985). R59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J. Biol. Chem.* **260**: 15762-15770.

Dennis, J., Waller, C., Timpl, R. & Schirrmacher, V. (1982). Surface sialic acid reduces attachment of metastatic tumor cells to collagen type IV and fibronectin. *Nature, (London)*, **300**: 274-276.

Depont, J.J.H.H.M., Van-Prooijen-van-Eeden, A. & Bonting, S.L.(1978). Role of negatively charged phospholipids in highly purified ($Na^+ + K^+$) ATP-ase from rabbit kidney outer medulla. Studies on ($Na^+ + K^+$)-activated ATPase. *Biochim. Biophys. Acta.* **508**: 464-477.

Derjagin, B.V. & Landau, L.D. (1941). Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. *Acta. Physicochemica. (USSR)*. 14: 633-662.

Diamond, L., O´Brien, T.G. & Baird, W..M. (1980). Tumor promoter and the mechanism of tumor promotion. *Adv. Cancer. Res.* **32**: 1-74.

Dinarello, C.A, Conti, P. & Mier, J.W. (1986). Effect of human interleukin-1 on natural killer cell activity: Is fever a host defense mechanism for tumor killing? *The yale journal of biology and medicine.* **59**: 97-106.

Dinarello, C.A., Marnoy, S.O. & Rosenwasser, L.J. (1983). Role of arachidonate metabolism in the immunoregulatory function of human leukocytic pyrogen/lymphocyte-activating factor/interleukin-1. *J. Immunol.* **130**: 890-895.

Dixit, V.M., Haverstick, D.M., O´Rourke, K.M., Hennessy, S.W., Grant, G.A., Santoro, S.A. & Frazier, W.A. (1985). A monoclonal antibody against human thrombospondin inhibits platelet aggregation. *Proc. Natl. Acad. Sci. (USA)*. **82**: 3472-3476.

Dolowy, K. (1980). A physical theory of cell-cell and cell substratum interactions. *In cell adhesion and motility,* Curtis, A.S.G. and Pitts, J.P. Eds. 3rd. Symposium of the british society for cell biology, Cambridge University Press, PP: 39-63.

Doroszewski, J., Skierski, J. & Przadka, L. (1977). Interaction of neoplastic cells with glass surface under flow conditions. *Expl. cell Res.* **104**: 335-343.

Dougherty, R.W. & Niedel, J.E. (1986). Cytosolic calcium regulates phorbol diester binding affinity in intact phagocytes. *J. Biol. Chem.* **261**: 4097-4100.

Droge, W. (1986). Protein kinase C in T-cell regulation. *Immunology today,* **7(11)**: 340-343.

Duband, J.L. & Thiery, J.P. (1984). Adhesive molecules and their role during the ontogeny of the peripheral nervous system. *In cellular and molecular control of direct cell interactions*, H.J. Marthy Ed. Plenum Press, New York and London, pp: 85-131.

Dunn, M.J., Owen, E. & Kemp, R.B. (1970). Studies on the mechanism underlying the inhibition by puromycin of cell aggregation in vitro. *J. Cell Sci.* **7**: 557-575.

Edelman, G.M. (1983). Cell adhesion molecules. *Science*, **219**: 450-457.

Edelman, G.M. (1984a). Cell adhesion and morphogenesis: The regulator hypothesis. *Proc. Natl. Acad. Sci. (USA)*. **81**: 1460-1464.

Edelman, G.M. (1984b). Cell adhesion molecules: A molecular basis for animal form. *Sci. Am.* **250**: 80-91.

Edelman, G.M. (1985). Expression of cell adhesion molecules during embryogenesis and regeneration. *Expl. cell Res.* **161**: 1-16.

Edelman, G.M., Gallin, W.J., Delouvee, A., Cunningham, B.A. & Thiery, J.P. (1983). Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. (USA)*. **80**: 4384-4388.

Edwards, J.G. & Campbell, J.A. (1971). The aggregation of trypsinised BHK 21 cells. *J. Cell Sci.* **8**: 53-72.

Ekblom, P., Vestweber, D. & Kemler, R.(1986). Cell-matrix interactions and cell adhesion during development. *Ann. Rev. Cell Biol.* **2**: 27-47.

Emmelot, P. (1977). *In mammalian cell membrane*, **vol. 2**, Jamieson, G.A. and Robinson, D.M., Eds. Butterworth & Co-publishers Ltd, London, pp. 1-51.

Engelhard, V.H., Esko,J., Sturm, D. & Glaser,M. (1976). Modification of adenylate cyclase activity in LM cells by manipulation of the

membrane phospholipid composition *in vivo*. *Proc. Natl. Acad. Sci. (USA)*. **73**: 4482-86.

Engvall, E. & Ruoslahti, E. (1977). Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer,* **20**: 1-5.

Erickson, H.P. & Bourdon, M.A. (1989). Tenascin: An extracellular matrix protein prominent in specialized embryonic tissues and tumors. Ann. Rev. Cell Biol. **5**: 71-92.

Europe-Finner, G.N. & Newell, P.C.(1985). Inositol 1,4,5-trisphosphate induces cyclic GMP formation in *Dictyostelium discoideum*. *Biophys. Biochem. Res.Commun*. **130**: 1115-1122.

Evans, P.M. & Lanham, D.F.M. (1986). Effects of inhibitors of arachidonic acid metabolism on intercellular adhesion of SV40-3T3 cells. *Cell Biol. Internatl. Reports,* **10**: 693-698.

Evans, W.H. (1987). Organelles and membranes of animal cells. *In biological membranes,* Findlay, J.B.C. & Evans, W.H. Eds. IRL Press Ltd. Oxford and Washington, DC. PP. 1-31.

Fantone, J.C. & Ward, P.A. (1982). Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *Am. J. Path*. **107**: 397-418.

Farquhar, M.G. & Palade, G.E. (1963). Junctional complexes in various epithelia. *J. Cell Biol*. **17**: 375-412.

Fischer, H., Ferber, E., Haupt, I., Kohlschutter, A., Modolell, M., Munder, P.G. & Sonak, R. (1967). Lysophosphatides and cell membranes. *Protides, Biol. Fluids,* **15**: 175-184.

Fisher, S.K., Van Rooijen, L.A.A. & Agranoff, B.W. (1984). Renewed interest in the polyphosphoinositides. *TIBS*. **9**: 53-56.

Fleischer, S., Bock, H.G. & Gazzotti, P.(1974). *In membrane proteins in transport and phosphorylation*, Azzone, G.F. Klingenberg, M.E., Quagliarello, E. & Siliprandi, N. Eds. North Holland Publishing Co., Amesterdam, pp.124-135.

Flower, R.J. (1975). Aspirin, prostaglandins, endoperoxides and platelets. *Nature, (London)*, **253**: 88-90.

Forrester, J.A., Ambrose, E.J. & Stoker, M.G.P. (1964). Micro-electrophoresis of normal and transformed clones of hamster kidney fibroblasts. *Nature, London*, **201**: 945-946.

Forrester, J.V. & Lakie, J.M. (1984). Adhesion of neutrophil leukocytes under conditions of flow. *J. Cell Sci.* **70**: 93-110.

Fourcans, B. & Jain, M.K.(1974). Role of phospholipids in transport and enzymic reactions. *Adv. Lipid. Res.* **12**: 147-226.

Frankfater, A. & Kuppy, T. (1981). Mechanism of association of N-acetyl-L-phenyl-alanylglycinal to papain. *Biochem.* 20: 5517-5524.

Frazier, W.A. (1987). Thrombospondin: A modular adhesive glycoprotein of platelets and nucleated cells. *J. Cell Biol.* **105**: 625-632.

Friend, D.S. & Gilula, N.B. (1972). Variations in tight and gap junctions in mammalian tissues. *J. Cell Biol.* **53**: 758-776.

Furie, M.B., Naprstek, B.L. & Silverstein, S.C. (1987). Migration of neutrophils across monolayers of cultured microvascular endothelial cells. An *in vitro* model of leukocyte extravasation. *J. Cell Sci.* **88**: 161-175.

Furlong, B., Henderson, A.H., Lewis, M.J. & Smith, J.A. (1987). Endothelium-derived relaxing factor inhibits *in vivo* platelet aggregation. *Br. J. Pharmacol.* **90**: 687-692.

Gallagher, J.T., Lyon, M. & Steward, W.P. (1986). Structure and function of heparin sulfate proteoglycans. *Biochem. J.* **236**: 313-325.

Gallatin, W.M., Weissman, I.L. & Butcher, E.C. (1983). A cell surface molecule involved in organ specific homing of lymphocytes. *Nature, (London)*, **304**: 30-34.

Gallin, J.I. (1980). Degranulating stimuli decrease the negative surface charge and increase the adhesiveness of human neutrophils. *J. Clin. Invest.* **65**: 298-306.

Gallin, W.J., Edelman, G.M. & Cunnigham, B.A.(1983). Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. *Proc. Natl. Acad. Sci. (USA)*. **80**: 1038-1042.

Gallin, W.J., Prediger, E.A., Edelman, G.M. & Cunnigham, B.A. (1985). Isolation of a cDNA clone for the liver cell adhesion molecule (L-CAM). *Proc. Natl. Acad. Sci. (USA)*. **82**: 2809-2813.

Gartner, T.K., Walz, D.A., Aiken, M., Starr-Spires, L. & Ogilivie, M.L., (1984). Antibodies against a 23 Kd heparin binding fragment of thrombospondin inhibit platelet aggregation. *Biochem. Biophys. Res. Commun.* **124**: 290-295.

Gaudry, M., Perianin, A., Marquetty, C. & J. Hakim. (1988). Negative effect of a protein kinase C inhibitor (H-7) on human polymorpho-nuclear neutrophil locomotion. *Immunology*, **63**: 715-719.

Gennaro, R., Florio, C. & Romeo, D. (1986). Co-activation of protein kinase C and NADPH oxidase in the plasma membrane of neutrophils cytoplasts. *Biochem. Biophys. Res. Commun.* **134**: 305-312.

George, J.N., Weed, R.I. & Reed, C.F.(1971). Adhesion of human erythrocytes to glass: The nature of the interaction and the effect of serum and plasma. *J. Cell physiol.* **77**: 51-60.

Gerisch, G. (1986). Interrelation of cell adhesion and differentiation in *Dictyostelium discoideum. J. Cell Sci. Suppl.* **4**: 201-209.

Gimbrone, M.A., Brock, A.F. & Schafer, A.I. (1984). Leukotrienne B_4 stimulates polymorphonuclear leukocyte adhesion to cultured vascular endothelial cells. *J. Clin. Invest.* **74**: 1552-1555

Gingell, D.& Todd, I. (1980). Red blood cell adhesion. II. Interferometric examination of the interaction with hydrocarbon oil and glass. *J. Cell Sci.* **41**: 135-149.

Gingell, D. & Vince, S. (1982). Substratum wettability and charge influence the spreading of *Dictyostelium* amoebae and the formation of ultrathin cytoplasmic lamellae. *J. Cell Sci.* **54**: 255-285.

Gingell, D., Todd, I. & Parsegian, V.A. (1977). Long range attraction between red cells and a hydrocarbon surface. *Nature, (London)*, **268**: 767-769.

Gingell, D. & Vince, S. (1980). Long-range forces and cell adhesion. *In cell adhesion and motility*, Curtis, A.S.G. & Pitts, J.P. Eds. 3rd. Symposium of the british society for cell biology, Cambridge University Press, PP. 1-37.

Goldstein, I.M., Malmsten, C.L., Kindahl, H., Kaplan, H.B., Radmark, O., Samuelson, B. & Weissmann, G. (1978). Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J. Expl. Med.* **148**: 787-792.

Goodenough, D.A. & Revel, J.P. (1970). A fine structural analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* **45**: 272-290.

Gordon, G.L. (1980). Mechanisms regulating platelet adhesion. In cell adhesion and motility, Curtis, A.S.G. and Pitts, J.P. Eds. 3rd. Symposium of the british society for cell biology, Cambridge University Press, pp. 199-233.

Goridis, C., De Agostini-Bazin, H., Hirn, M., Hirsch, M.R., Rougon, G., Sadoul, R., Langley, O.K., Gombos, G. & Finne, J. (1984). Neural surface antigens during nervous system development. *In cold spring harbor symposia on quantitative biology.* Vol. **48**: 527-537.

Gorman, R.R., Bunting, S. & Miller, O.V. (1977). Modulation of human platelet adenylate cyclase by prostacyclin. *Prostaglandins*, **13**: 377-388.

Gorter, E. & Grendel, F. (1925). On bimolecular layers of lipoids on the chromocytes of the blood. *J. Expl. Med.* **41**: 439-443.

Graf, J., Iwamoto, Y., Sasaki, M., Martin, G.R., Kleinman, H.K., Robey, F.A. & Yamada, Y. (1987). Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis and receptor binding. *Cell*, **48**: 989-996.

Grant, L. (1973). The sticking of white blood cells in inflammation. *In the inflammatory process, vol: II,* Zweifach, B.M., Grant, L.& McCluskey, R. Eds. New York, London, Academic press, PP: 205-249.

Griepp, E,B., Dolan, W.J., Robbins, E.S. & Sabatini, D.D. (1983). Participation of plasma membrane proteins in the formation of tight junctions by cultured epithelial cells. *J. Cell Biol.* **96**: 693-702.

Grinnell, F. (1976). The serum dependence of baby hamster kidney cell attachment to a substratum. *Expl. Cell Res.* **97**: 265-274.

Grinnell, F. (1978). Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* **53**: 65-144.

Grinnell, F. (1984). Fibronectin and wound healing. *J. Cell. Biochem.* **26**: 107-116.

Grinnell, F. & Feld, M.K. (1979). Initial adhesion of human fibroblast in serum-free medium: possible role of secreted fibronectin. *Cell*, **17**: 117-129.

Gros, D.B., Nicholson, B.J. & Revel, J.P. (1983). Comparative analysis of the gap junction protein from rat heart and liver: Is there a tissue specificity of gap junctions? *Cell*, **35**: 539-549.

Gruber, M.Y., Cheng, K.-H., Lepock, J.R. & Thompson, J.E. (1984). Improved yield of plasma membrane from mammalian cells through modifications of the two phase polymer isolation procedure. *Anal. Biochem.* **138**: 112-118.

Grumet, M., Hoffman, S. & Edelman, G.M. (1984a). Two antigenically related neuronal cell adhesion molecules of different specificities mediate neuron- neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. (USA)*. **81**: 267-271.

Grumet, M. Hoffman, S., Chuong, C-M. & Edelman, G.M. (1984b). Polypeptide components and binding functions of neuron-glia cell adhesion molecule. *Proc. Natl. Acad. Sci. (USA)*. **81**: 7989-7993.

Grumet, M. & Edelman, G.M. (1984). Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. cell Biol.* **98**: 1746-1756.

Guidotti, G. (1972). Membrane proteins. *Ann. Rev. Biochem.* **41**: 731-752.

Hallam, T.J., Sanchez, A. & Rink, T.J. (1984). Stimulus-response coupling in human platelets. Changes evoked by platelet-activating factor in cytoplasmic free calcium monitored with the fluorescent calcium indicator Quin 2. *Biochem. J.* **218**: 819-827.

Hamberg, M., Svensson, J. & Samuelsson, B. (1975). Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. (USA)*. **72**: 2994-2998.

Hansen, O.C., Nybroe, O. & Bock, E. (1985). Cell-free synthesis of the D2-cell adhesion molecule: Evidence for three primary translation products. *J. Neurochem.* **44**: 712-717.

Harlan, J. (1985). Leukocyte-endothelial interactions. *Blood J. Haematol.* **65**: 513-525.

Harlan, J.M., Killen, P.D., Senecal, F.M., Schwartz, B.R., Yee, E.K., Taylor, R.F., Beatty, P.G., Price, T.H. & Ochs, H.D. (1985). The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium *in vitro* . *Blood, J. Hematology*, **66**: 167-178.

Harris, A. (1973). Behaviour of cultured cells on substrata of variable adhesiveness. *Expl. cell Res.* **77**: 285-297.

Haskard, D., Cavender, D. & Ziff, M. (1986). Phorbol ester-stimulated T-lymphocytes show enhanced adhesion to human endothelial cell monolayers. *J. Immunol.* **137**: 1429-1434.

Hawley, H. P. & Gordon, G.B. (1976). The effect of long chain free fatty acids on human neutrophil function and structure. *Lab. Invest.* **34**: 216-222.

Hayakawa, T., Suzuki, K., Suzuki, S., Andrews, P.C. & Babior, B.M. (1986). A possible role for protein phosphorylation in the activation of the respiratory burst in human neutrophils. *J. Biol. Chem.* **261**: 9109-9115.

Hemler, M.E. (1988). Adhesive protein receptors on hematopoietic cells. *Immunol. Today*, **9** (4): 109-113.

Hesketh, T.R., Smith, G.A., Houslay, M.D., Warren, G.B.& Metcalfe, J.C. (1977). Is an early calcium flux necessary to stimulate lymphocytes? *Nature, (London)*, **267**: 490-494.

Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.W. & Metcalfe, J.C. (1983). Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. *J. Biol. Chem.* **258**: 4876-4882.

Higgs, E.A., Moncada, S. & Vane, J.R. (1979). The role of arachidonic acid metabolites in inflammation. *In advances in inflammation research*, **Vol. 1**, Weissmann, G., Samuelsson, B.& Paoletti, R. Eds. Raven press, New York, PP. 413-418.

Higgs, E.A., Moncada, S., Vane, J.R., Caen, J.P. Michel, H. & Tobelem, G. (1978). Effect of prostacyclin (PGI_2) on platelet adhesion to rabbit subendothelium. *Prostaglandins*, **16**: 17-22.

Hirata, F., Corcoran, B.A., Venkatasubramanian, K., Schiffmann, E. & Axelrod, J. (1979). Chemoattractants stimulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes. *Proc. Natl. Acad. Sci. (USA)*. **76**: 2640-2643.

Hoffman, S., Sorkin, B.C., White, P.C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B.A. & Edelman, G.M. (1982). Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* **257**: 7720-7729.

Hokin, M.R. & Hokin, L.E. (1953). Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. *J. Biol. Chem.* **203**: 967-977.

Hoover, R.L. (1978). Modulation of the cell surface and the effects on cellular interactions. *In cell-cell recognition*, A.S.G. Curtis Ed. Symposia of the society for experimental biology, **32**, Cambridge University Press, pp. 221-240.

Hoover, R. L., Lynch, R. & Karnovsky, M. J. (1977). Decrease in adhesion of cells cultured in polyunsaturated fatty acids. *Cell*, **12**: 295-300.

Hoover, R.L., Briggs, R.T. & Karnovsky, M.J. (1978). The adhesive interaction between polymorphonuclear leukocytes and endothelial cells *in vitro*. *Cell*, **14**: 423-428.

Hoover, R.L., Folger, R., Haering, W.A., Ware, B.R. & Karnovsky, M.J. (1980). Adhesion of leukocytes to endothelium: Roles of divalent cations, surface charge, chemotactic agents and substrate. *J. cell Sci.* **45**: 73-86.

Hoover, R.L., Karnovsky, M.J. Austen, K.F., Corey, E.J. & Lewis, R.A. (1984). Leukotriene B_4 action on endothelium mediates augmented

neutrophil/ endothelial adhesion. *Proc. Natl. Acad. Sci. (USA)*. **81**: 2191-2193.

Hoover, R.L., Robinson, J.M. & Karnovsky, M.J. (1987). Adhesion of polymorphonuclear leukocytes to endothelium enhances the efficiency of detoxification of oxygen-free radicals. *Am. J. Pathol.* **126**: 258-268.

Horwitz, A,F., Hatten, M.E. & Burger, M.M. (1974). Membrane fatty acid replacements and their effect on growth and lectin-induced agglutinability. *Proc. Natl. Acad. Sci. (USA)*. **71**: 3115-3119.

Houslay, M.D., Hesketh, T.R., Smith, G.A., Warren, G.B. & Metcalfe, J.C. (1976). The lipid environment of the glucagon receptor regulate adenylate cyclase activity. *Biochim. Biophys. Acta*. **436**: 495-404.

Hsie, A.W. & Puck, T.T. (1971). Morphological transformation of chinese hamster cells by dibutyryl adenosine cyclic 3′:5′-monophosphate and testosterone. *Proc. Natl. Acad. Sci. (USA)*. **68**: 358-361.

Humphreys,T. (1963). Chemical dissolution and *in vitro* reconstruction of sponge cell adhesion: I. Isolation and functional demonstration of the components involved. *Devel. Biol.* **8**: 27-47.

Hyafil, F., Babinet, C. & Jacob, F. (1981). Cell-cell interactions in early embryogenesis: A molecular approach to the role of calcium. *Cell*, **26**: 447-454.

Hyafil, F., Morello, D., Babinet, C. & Jacob, F. (1980). A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos. *Cell*, **21**: 927-934.

Hynes, R.O. (1987). Integrins: A family of cell surface receptors. *Cell*, **48**: 549-554.

Jaffe, E.A., Ruggiero, J.T., Leung, L.L.K. & Doyle, M.J. (1983). Cultured human fibroblasts synthesize and secrete thrombospondin and

incorporate it into extracellular matrix. *Proc. Natl. Acad. Sci. (USA)*. **80**: 998-1002.

Johns, R.A. & Peach, M.J. (1987). *para*-Bromophenacyl bromide inhibits endothelium-dependent arterial relaxation and cyclic GMP accumulation by effects produced exclusively in the smooth muscle. *J. Pharmacol. Expl. Therapy,* **244**: 859-865.

Johnson, G.S., Friedman, R.M. & Pastan, I. (1971). Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3′:5′-cyclic monophosphate and its derivatives. *Proc. Natl. Acad. Sci. (USA)*. **68**: 425-429.

Johnson, G.S., Morgan, W.D. & Pastan, I. (1972). Cyclic AMP increases the adhesion of fibroblasts to substratum. *Nat.New Biol*. **236**: 247-249.

Jones, G.E. (1974). Intercellular adhesion: Modification by dielectric properties of the medium. *Membrane Biology* , **16**: 297-312.

Jones, J.C.R. & Goldman, R.D. (1985). Intermediate filaments and the initiation of desmosome assembly. *J. Cell Biol.* **101**: 506-517.

Jorgensen, O.S., Delouvee, A., Thiery, J.P. & Edelman, G.M. (1980). The nervous system specific protein D2 is involved in adhesion among neurites from cultured rat ganglia. *FEBS. Lett.* **111**: 39-42.

Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. & Williamson, J.R. (1984). *myo*-Inositol 1,4,5-trisphosphate. A second messenger for the hormonal mobilization of intracellular Ca^{2+} in liver. *J. Biol. Chem.* **259**: 3077-3081.

Kachar, B. & Rees, T.S. 1982. Evidence for the lipidic nature of tight junction strands. *Nature. (London)*, **296**: 464-466.

Keller, H.U., Niggli, V. & Zimmermann, A. (1990). The protein kinase C inhibitor H-7 activates human neutrophils: effect on shape, actin polymerization, fluid pinocytosis and locomotion. *J. Cell Sci.* **96**: 99-106.

Keller, H.U., Wissler, J.H. & Damerau, B. (1981). Diverging effects of chemotactic serum peptides and synthetic f-met-leu-phe on neutrophil locomotion and adhesion. *Immunology*, **42**: 379-383.

Kelly, D.E., (1966). Fine structure of desmosomes, hemidesmosomes, and adepidermal globular layer in developing newt epidermis. *J. Cell Biol.* **28**: 54-72.

Kemp, R.B. (1970). The effect of neuraminidase (3:2:1:18) on the aggregation of cells dissociated from embryonic chick muscle tissue. *J. Cell Sci.* **6**: 751-766.

Kikkawa, U. & Nishizuka, Y. (1986). The role of protein kinase C in transmembrane signalling. *Ann. Rev. Cell Biol.* **2**: 149-178.

Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980). Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidyl-inositol turnover. *J. Biol. Chem.* **255**: 2273-2276.

Kleinman, H.K., Klebe, R.J. & Martin, G.R. (1981). Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* **88**: 473-485.

Kleinman, H.K., Martin, G.R. & Fishman, P.H. (1979). Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. (USA).* **76**: 3367-3371.

Kolodny, G.M. (1972). Effect of various inhibitors on readhesion of trypsinized cells in culture. *Expl. Cell Res.* **70**: 196-202.

Kondo, K. & Sakai, H.(1971). Demonstration and preliminary characterization of reaggregation-promoting substances from embryonic sea urchin cells. *Devel. Growth Diff.* **13**: 1-14.

Kondo, S., Kawamura, K., Iwanaga, J., Hamada, M., Aoyagi, T., Maeda, K., Takeuchi, T. & Umezawa, H. (1969). Isolation and characterization

of leupeptins produced by actinomycetes. *Chem. Pharm. Bull.* **17**: 1896-1901.

Korchak, H.M., Vienne, K., Rutherford, L.E., Wilkenfeld, C., Finkelstein, M.C. & Weissmann, G. (1984). Stimulus response coupling in the human neutrophil. II. Temporal analysis of changes in cytosolic calcium efflux. *J. Biol. Chem.* **259**: 4076-4082.

Korchak, H.M., Vosshall, L.B., Hainess, K.A., Wilkenfeld, C., Lundquist, K.F. & Weissmann, G. (1988b). Activation of the human neutrophil by calcium-mobilizing ligands. II. Correlation of calcium, diacylglycerol and phosphatidic acid generation with superoxide anion generation. *J. Biol. Chem.* **263**: 11098-11105.

Korchak, H.M., Vosshall, L.B., Zagon, G., Ljubich, P., Rich, A.M. & Weissmann, G. (1988a). Activation of the neutrophil by calcium-mobilizing ligands. I. A chemotactic peptide and the lectin concanavalin A stimulate superoxide anion generation but elicit different calcium movements and phosphoinositide remodeling. *J. Biol. Chem.* **263**: 11090-11097.

Kramer, C., Franson, R. & Rubin, R. (1984). Regulation of phosphatidyl inositol turnover, calcium metabolism and enzyme secretion by phorbol dibutyrate in neutrophils. *Lipids.* **19 (5)**: 315-323.

Kramer, R.H. & Nicolson, G.L. (1979). Interaction of tumour cells with vascular endothelial cell monolayers: A model for metastatic invasion. *Proc. Natl. Acad. Sci. (USA).* **76**: 5704-5708.

Kreisberg, J.I., Venkatachalam, M.A. & Patel, P.Y. (1984). Cyclic AMP-associated shape change in mesangial cells and its reversal by prostaglandin E_2. *Kidney International*, **25**: 874-879.

Kuno, M. & Gardner, P. (1987). Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature, (London)*, **326**: 301-304.

Lapetina, E.G. & Cuatrecasas, P. (1979). Stimulation of phosphatidic acid production in platelets precedes the formation of arachidonate and parallels the release of serotonin. *Biochem. Biophys. Acta.* **573**: 394-402.

Lapetina, E.G., Billah, M.M. & Cuatrecasas, P. (1981). The initial action of thrombin on platelets. Conversion of phosphatidylinositol to phosphatidic acid preceding the production of arachidonic acid. *J. Biol. Chem.* **256**: 5037-5040.

Lawler, J.W., Slayter, H.S. & Coligan, J.E. (1978). Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J. Biol. Chem.* **253**: 8609-8616.

Lee, S., Gilula, N.B. & Warner, A.E. (1987). Gapjunctional communication and compaction during preimplantation stages of mouse development. *Cell*, **51**: 851-860.

Leivo, I., Vaheri, A., Timpl, R. & Wartivaara, J. (1980). Appearance and distribution of collagens and laminin in the early mouse embryo. *Devel. Biol.* **76**: 100-114.

Levitzki, A. (1980). Quantitative aspects of ligand binding to receptors. *In cellular receptors*, D. Schulster & A. Levitzki, Eds. pp.16-17.

Lew, P-D., Dayer, J.M., Wollheim, C.B. & Pozzan, T. (1984). Effect of leukotriene B_4, prostaglandin E_2 and arachidonic acid on cytosolic free calcium in human neutrophils. *FEBS. Lett.* **166**: 44-48.

Lilien, J.E. & Moscona, A.A. (1967). Cell aggregation: Its enhancement by a supernatant from cultures of homologous cells. *Science*, **157**: 70-72.

Lindner, L., Rathjen, F.G. & Schachner, M. (1983). L-1 mono and polyclonal antibodies modify cell migration in early post natal mouse cerebellum. *Nature, (London)*, **305**: 427-430.

Lloyd, C.W. & Cook, G.M.W. (1974). On the mechanism of the increased aggregation by neuraminidase of 16 C malignant rat dermal fibroblasts in vitro. *J. Cell Sci.* **15**: 575-590.

MaCfarlane, R.G. (1976). *In human blood coagulation, haemostasis and thrombosis*, Biggs, R.M. Ed. Blachwell Scientific Publications, pp. 608-654.

MacGregor, R.R., Macarak, E.J. & Kefalides, N.A. (1978). Comparative adherence of granulocytes to endothelial monolayers and nylon fiber. *J. Clin. Invest.* **61**: 697-702.

MacIntyre, D.E. & Gordon, J.L. (1974). Prostaglandin receptor on blood platelets: Effect of thiol reagents on inhibition of platelet aggregation by prostaglandin E_1. *Biochem. Soc. Transaction.* **2**: 1265-1269.

MacIntyre, D.E. & Gordon, J.L. (1975). Calcium-dependent stimulation of platelet aggregation by prostaglandin E_2. *Nature, (London)*, **258**: 337-339.

Malech, H.L., Root, R.K. & Gallin, J.I. (1977). Structural analysis of human neutrophil migration. *J. Cell Biol.* **75**: 666-693.

Margolis, L.B., Dyatlovitskaya, E.V. & Bergelson, L.D. (1978). Cell lipid interaction. Cell attachment to lipid substrates. *Expl. Cell Res.* **111**: 454-457.

Marone, G., Thomas, L.L. & Lichtenstein, L.M. (1980). The role of agonists that activate adenylate cyclase in the control of cAMP metabolism and enzyme release by human polymorphonuclear leukocytes. *J. Immunol.* **125**: 2277-2283.

Maroudas, N.G. (1973). Chemical and mechanical requirements for fibroblast adhesion. *Nature, (London)*, **244**: 353-354.

Maroudas, N.G. (1977).Sulphonated polystyrene as an optimal substratum for the adhesion and spreading of mesenchymal cells in monovalent and divalent saline solutions. *J. Cell. Physiol.* **90**: 511-520.

Martin, G.R. & Rubin, H. (1974). Effects of cell adhesion to the substratum on the growth of chick embryo fibroblasts. *Expl. Cell Res.* **85**: 319-333.

Martin, G.R. & Timpl, R. (1987). Laminin and other basement membrane components. *Ann. Rev. cell Biol.* **3**: 57-85.

Mazurov, A.V., Misselwitz, F., Hoffman, U., Leytin, V.L. & Repin, V.S. (1988). Effects of prostacyclin analogue (carbacyclin) on the interaction of platelets with different collagen substrates. Inhibition of cAMP increase by collagen type I, III and IV. *Prostaglandins*, **35**: 51-65.

McClain, D.A. & Edelman, G.M. (1982). A neural cell adhesion molecule from human brain. *Proc. Natl. Acad. Sci. (USA)*. **79**: 6380-6384.

McClay, D.R. & Ettensohn, C.A. (1987). Cell adhesion in morphogenesis. *Ann. Rev. Cell Biol.* **3**: 319-345.

McCrea, J.M., Robinson, P.& Gerrard, J.M. (1985). Mepacrine (quinacrine) inhibition of thrombin-induced platelet responses can be overcome by lysophosphatidic acid. *Biochim. Biophys. Acta.* **842**: 189-194.

McGillen, J., Patterson, R. & Phair, J.P. (1980). Adherence of polymorphonuclear leukocytes to nylon: Modulation by prostacyclin (PGI_2), corticosteroids, and complement activation. *J. Infectious Dis.* **141**: 382-388.

McInnes, C., Knox, P. & Winterbourne, D.J. (1987). Cell spreading on serum is not identical to spreading on fibronectin. *J.Cell Sci.* **88**: 623-629.

McIntyre, T.M., Zimmerman, G.A. & Prescott, S.M. (1986). Leukotrienes C_4 and D_4 stimulate human endothelial cells to synthesize platelet-activating factor and bind neutrophils. *Proc. Natl. Acad. Sci. (USA).* **83**: 2204-2208.

McKean, M.L., Smith, J.B. & Silver, M.J. (1981). Formation of lysophosphatidyl- choline by human platelets in response to thrombin. Support for the phospholipase A_2 pathway for the liberation of arachidonic acid. *J. Biol. Chem.* **256**: 1522-1524.

Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. & Horecker, B.L. (1985). Binding of protein kinase C to neutrophil membranes in the presence of calcium and its activation by a calcium-requiring proteinase. *Proc. Natl. Acad. Sci. (USA).* **82**: 6435-6439.

Michell, R.H., (1975). Inositolphospholipids and cell surface receptor function. *Biochim, Biophy. Acta.* **415**: 81-147.

Mishell, B.B. & Shiigi, S.M. (1980). Selected methods in cellular immunology. W.H. Freeman and Company, USA.

Moncada, S., Gryglewski, R., Bunting, S. & Vane, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature, (London),* **263**: 663-665.

Monsigny, M., Kieda, C. & Roche, A-C. (1983). Membrane glycoproteins, glycolipids and membrane lipids as recognition signals in normal and malignant cells. *Biol. Cell,* **47**: 95-110.

Moscona, A. (1961). Effect of temperature on adhesion to glass and histogenetic cohesion of dissociated cells. *Nature, (London),* **190**: 408-409.

Moscona, A. (1962). Analysis of cell recombinations in experimental synthesis of tissues *in vitro*. *J. Cell Comp. Physiol., Suppl.* 1, **60**: 65-80.

Moscona, A. (1963). Inhibition by trypsin inhibitors of dissociation of embryonic tissue by trypsin. *Nature, (London)*, **199**: 379-380.

Moscona, M. & Moscona, A. (1966). Inhibition of cell aggregation in vitro by puromycin. *Expl. Cell Res.* **41**: 703-706.

Mueller, H. & Franke, W.W. (1983). Biochemical and immunological characterization of desmoplakins I and II, the major polypeptides of desmosomal plaque. *J. Mol. Biol.* **163**: 647-671.

Muid, R.E., Penfield, A. & Dale, M.M. (1987). The diacylglycerol kinase inhibitor R59 022, enhances the superoxide generation from human neutrophils induced by stimulation of fMeth-Leu-Phe., IgG and C3b receptors. *Biochem. Biophys. Res. Commun.* **143**: 630-637.

Murota, S., Chang, W-C., Tsurufuji, S. & Morita, I. (1979). The possible roles of prostacyclin (PGI_2) and thromboxane in chronic inflammation. *In: Advances in inflammation research,* Weissmann, G. Samuelsson, B. & Paoletti, R. Eds. **Vol.1**: 439-455. Raven Press, New York.

Nagao, S., Kitajima, Y., Nagata, K., Inoue, S., Yaoita, H. & Nozawa, Y. (1989). Correlation between cell-cell contact formation and activation of protein kinase C in human squamous cell carcinoma cell line. *J. Invest. Dermatol.* **92**: 175-178.

Nakatsuji, N. (1986). Presumptive mesoderm cells from *Xenopus Laevis* gastrulae attach to and migrate on substrata coated with fibronectin or laminin. *J. Cell Sci.* **86**: 109-118.

Nicholson, B.J., Takemoto, L.J., Hunkapiller, M.W., Hood, L.E. & Revel, J-P. (1983). Differences between liver gap junction protein and lens MIP 26 from rat: Implications for tissue specificity of gap junctions. *Cell*, **32**: 967-978.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature (London)*, **308**: 693-698.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science*, **233**: 305-312.

Norris, W.D., Steele, J.G., Johnson, G. & Underwood, P.A. (1990). Serum enhancement of human endothelial cell attachment to and spreading on collagen I and IV does not require serum fibronectin or vitronectin. *J. Cell Sci.* **95**: 255-262.

Nunn, D.L. & Watson, S.P. (1987). A diacylglycerol kinase inhibitor, R59 022, potentiates secretion by and aggregation of thrombin-stimulated human platelets. *Biochem. J.* **243**: 809-813.

Oldfield, E. & Chapman, D. (1972). Dynamics of lipids in membranes: heterogeneity and the role of cholesterol. *FEBS. Lett.* **23**: 285-297.

Oseas, R., Young, H-H., Baehner, R.L. & Boxer, L.A. (1981). Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. *Blood*, **57**: 939-945.

Ostergaard, H. & Clark, W.R. (1987). The role of calcium in activation of mature cytotoxic T-lymphocytes for lysis. *J. Immunol.* **139**: 3573-3579.

Otto, A.M., Nilsen-Hamilton, M., Boss, B.D., Ulrich, M-O. & Jimenez De Asua, L. (1982). Prostaglandin E_1 and E_2 interact with prostaglandin F_{2a} to regulate initiation of DNA replication and cell division in Swiss 3T3 cells. *Proc. Natl. Acad. Sci. (USA).* **79**: 4992-4996.

Owens, N.F., Gingell, D. & Trommler, A. (1988). Cell adhesion to hydroxyl groups of a monolayer film. *J.Cell Sci.* **91**: 269-279.

O'Flaherty, J.T., Kreutzer, D.L. & Ward, P.A. (1979a). Effect of prostaglandin E_1, E_2 and $F_{2\alpha}$ on neutrophil aggregation. *Prostaglandins*, **17**: 201-210.

O´Flaherty, J.T., Kreutzer, D.L. & Ward, P.A. (1979b). Effect of cytochalasin B on human neutrophil aggregation. *Expl. Cell Res.* **120**: 31-37.

O´Rourke, F.A., Halenda, S.P., Zavoico, G.B. & Feinstein, M.B. (1985). Inositol 1, 4, 5-trisphosphate release Ca^{2+} from a Ca^{2+}-transporting membrane vesicle derived from human platelets. *J. Biol. Chem.* **260**: 956-962.

Parish, R.W., Schmidhauser, C., Schmiot, T. & Dudler, R. (1987). Mechanisms of tumor cell metastasis. In cell behavior: Shape, adhesion and motility. *J. Cell Sci. Suppl.* **8**: 181-197.

Parsegian, V.A. & Gingell, D. (1972). On the electrostatic interaction across a salt solution between two bodies bearing unequal charge. *Biophys. J.* **12**: 1192-1204.

Parsegian, V.A. & Gingell, D. (1980). Red blood cell adhesion. III: Analysis of forces. *J. Cell. Sci.* **41**: 151-157.

Pearson, J.D., Carleton, J.S., Beesley, J.E., Hutchings, A. & Gordon, J.L. (1979). Granulocyte adhesion to endothelium in culture. *J. Cell Sci.* **38**: 225-235.

Peracchia, C. (1973). Low resistance junctions in cray fish. II. Structural details and further evidence for intercellular channels by freeze-fracture and negative staining. *J. Cell Biol.* **57**: 66-76.

Pitts J.D., Finbow, M. E., Eldridge, T., Bultjens, J., Kan, E. & Shuttleworth, J. (1985). Junctional communication and communication compartments in development. *In cellular and molecular contact of direct cell interactions*, Marthy, H.-J. Ed. V: **99**, Plenum Press, New York. pp. 323-335.

Poste, G. (1971). Tissue dissociation with proteolytic enzymes at the cell surface. *Expl. Cell Res.* **65**: 359-367.

Prentki, M., Corkey, B.E. & Matschinsky, F.M. (1985). Inositol 1, 4, 5-tris phosphate and the endoplasmic reticulum calcium cycle of a rat insulinoma cell line. *J. Biol. Chem.* **260**: 9185-9190.

Prentki, M., Wollheim, C.B. & Lew, P.D. (1984). Calcium homeostasis in permeabilized human neutrophils. Characterization of calcium-sequestering pools and the action of inositol 1, 4, 5-trisphosphate. *J. Biol. Chem.* **259**: 13777-13782.

Prives, J. & Shinitzky, M. (1977). Increased membrane fluidity precedes fusion of muscle cells. *Nature (London)*, **268**: 761-763.

Pytela, R., Pierschbacher, M.D. & Ruoslahti, E. (1985a). A 125/115-KDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. *Proc. Natl. Acad. Sci. (USA)*. **82**: 5766-5770.

Pytela, R., Pierschbacher, M.D.& Ruoslahti, E. (1985b). Identification and isolation of a 140 Kd cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell*, **40**: 191-198.

Rabinovitch, M. & De-Stefano, M.J. (1973). Manganese stimulates adhesion and spreading of mouse sarcoma I ascites cells. *J. Cell Biol.* **59**: 165-176.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1987). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br. J. Pharmacol.* **92**: 181-187.

Ramstedt, U., Ng, J., Wigzell, H., Serhan, C.N. & Samuelson, B. (1985). Action of novel eicosanoids lipoxin A and B on human natural killer cell cytotoxicity: Effects on intracellular cAMP and target cell binding. *J. Immunol.* **135**: 3434-3438.

Rao, K.M.K. (1985). Phorbol esters and retinoids induce actin polymerization in human leukocytes. *Cancer Lett.* **28**: 253-262.

Revel, J.P. & Karnovsky, M.J. (1967). Hexagonal array of subunits in intracellular junctions of the mouse heart and liver. *J. Cell Biol.* **33:** C7-C12.

Richmond, J.E., Glaeser, R.M. & Todd, P. (1968). Protein synthesis and aggregation of embryonic cells. *Expl. Cell Res.* **52:** 43-58.

Rink, T.J., Smith, S.W. & Tsien, R.Y. (1982). Cytoplasmic free calcium in human platelets: Calcium thesholds and calcium-independent activation for shape-change and secretion. *FEBS. Lett.* **148:** 21-26.

Roberts, M.F., Deems, R.A., Mincey, T.C. & Dennis, E.A. (1977). Chemical modification of the histidine residue in phospholipase A_2 (Naja, naja, naja). *J. Biol. Chem.* **252:** 2405-2411.

Robertson, J.D. (1981). Membrane structure. *J. Cell Biol.* **91:** 189s-204s.

Roseman, S. (1970). The synthesis of complex carbohydrates by multiglycosyl-transferase systems and their potential function in intercellular adhesion. *Chem. Phys. Lipids*, **5:** 270-297.

Roth, S. (1968). Studies on intercellular adhesive selectivity. *Devl. Biol.* **18:** 602-631.

Roth, S., McGuire, E.J. & Roseman, S. (1971). An assay for intercellular adhesive specificity. *J. Cell Biol.* **51:** 525-535.

Rothfield, L.I. & Romeo, D. (1971). Enzyme reactions in biological membranes. *In structure and function of biological membrane*, L.I. Rothfield Ed. Academic Press. Inc. New York and London Ltd., pp. 251-283.

Rubin, R.P., Sink, L.E. & Freer, R.G. (1981). On the relationship between fMet-Leu-Phe stimulation of arachidonyl phosphatidylinositol turnover and lysosomal enzyme secretion by rabbit neutrophils. *Mol. Pharmacol.* **19:** 31-37.

Rutherford, N.G. & Cook, G.M.W. (1981). Isolation and characterization of mannan-binding protein associated with the early chick embryo. *FEBS. Lett.* **136**: 105-110.

Sandermann, H. (1978). Regulation of membrane enzymes by lipids. *Biochim. Biophys. Acta*. **515**: 209-237.

Schaeffer, B.E. & Curtis, A.S.G. (1977). Effect on cell adhesion and membrane fluidity of changes in plasmalemmal lipids in mouse L929 cells. *J. Cell Sci.* **26**: 47-55.

Schafer, A.I., Cooper, B., O'Hara, O. & Handin, R.I. (1979). Identification of platelets receptors for prostaglandin I_2 , D_2. *J. Biol. Chem.* **254**: 2914-2917.

Seaman, G.V.F. (1973). The surface chemistry of the erythrocyte and thrombocyte membrane. *J. Supra-mol. Struct.* **1**: 437-447.

Shaw, J.O. (1980). Leukocytes in chemotactic-fragment-induced lung inflammation: Vascular emigration and alveolar surface migration. *Am. J. Pathol.* **101**: 283-302.

Shaw, L.M., Messier, J.M. & Mercurio, A.M. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the a6b1 integrin. *J.Cell Biol.* **110**: 2167-2174.

Shaw, R.J., Walsh, G.M., Cromwell, O., Moqbel, R., Spry, C.J.F. & Kay, A.B. (1985). Activated human eosinophils generate SRS-A leukotrienes following IgG-dependent stimulation. *Nature (London)*, **316**: 150-152.

Shell-Frederick, E. (1984). A comparison of the effects of soluble stimuli on free cytoplasmic and membrane bound calcium in human neutrophils. *Cell calcium*, **5**: 237-251.

Simpson, L.O. (1988). Altered blood rheology in the pathogenesis of diabetic and other neuropathies. *Muscle Nerve*, **11**: 725-744.

Singer, S.J. & Nicolson, G.L. (1972). The fluid mosaic model of the structure of cell membranes. *Science*, **175**: 720-731.

Snyderman, R. & Pike,M.C. (1984). Regulation of leukocyte transductional mechanisms of chemoattractants receptors on leukocytes. *Ann. Rev. Immunol.* **2**: 257-281.

Spagnuolo, P.J., Ellner, J.J., Hassid, A. & Dunn, M.J. (1980). Thromboxane A_2 mediates augmented polymorphonuclear leukocyte adhesiveness. *J. Clin. Invest.* **66**: 406-414.

Spat, A., Fabiato, A. & Rubin, R.P. (1986). Binding of inositol trisphosphate by microsomal fraction. *Biochem. J.* **233**: 929-932.

Spector, A.A. (1975). Fatty acid binding to plasma albumin. *J.Lipid Res.* **16**: 165-

Staehelin, L.A. (1974). Structure and function of intercellular Junctions. *Int. Rev. Cytol.* **39**: 191-283.

Staltz, J.F., Prost-Dvojabovic, R.F., Streiff, F., Samana, M.& Larcan, A. (1973). Influence d´ acides gras saturés et non saturés sur la pression de filtration de plasma riche en plaquettes. Correlation avec l´aggregation plaquettaire. *Acta. Haemat.* **49**: 306-313.

Stotboom, A.J., Verheij, H.M. & Dehaas, G.A. (1982). On the mechanism of phospholipase A_2. *In phospholipids*, **vol. 4**, Hawthorn, J.N. & Ansell, J.B. Eds. Elsivier Biomedical Press, pp. 359-434

Streb, H., Irvine, R.F., Berridge, M.J. & Schulz, I. (1983). Release of calcium from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. *Nature (London)*, **306**: 67-69.

Swift, D.L. & Friendlander, S.K. (1964). The coagulation of hydrosols by brownian motion and laminar shear flow. *J. Colloid. Sci.* **19**: 621-647.

Takada, Y., Wayner, E.A., Karter, W.G. & Hemler, M.E. (1988). Extracellular matrix receptors ECMR II and ECMR I for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. *J. Cell Biochem.* **37**: 385-393.

Takeichi, M. (1971). Changes in the properties of cell-substrate adhesion during cultivation of chicken fibroblasts *in vitro* in a serum-free medium. *Expl. Cell Res.* **68**: 88-96.

Takeichi, M., Yoshida-Noro, C., Ogou, S., Shirayoshi, Y. & Okada, T.S. (1983). A cell-cell adhesion molecule involved in embryonic cellular interactions as studied by using teratocarcinoma cells. *In teratocarcinoma stem cells*, Silver, L.M., Martin, G.R. & Strickland, S. Eds. Cold Spring Harbor, Mass: Cold Spring Harbor Lab. pp. 163-171.

Takenawa, T., Ishitoya, J. & Nagi, Y. (1986). Inhibitory effect of prostaglandin E_2, Forskolin, and dibutyryl cAMP on arachidonic acid release and inositol phospholipid metabolism in guinea pig neutrophils. *J. Biol. Chem.* **261**: 1092-1098.

Terranova, V.P., Hujanen, E.S. & Martin, G.R. (1986). Basement membranes and the invasive activity of metastatic tumor cells. *J. Natl. Cancer Inst.* **77**: 311-316.

Terranova, V.P., Liotta., L.A., Russo, R.G. & Martin, G.R. (1982). Role of laminin in the attachment and metastasis of murine tumor cells. *Cancer Res.* **42**: 2265-2269.

Tevelde, A.A., Keizer, G.D. & Figdor, C.G. (1987). Differential function of LFA-1 family molecules (CD11 and CD18) in adhesion of human monocytes to melanoma and endothelial cells. *Immunol.* **61**: 261-267.

Thiery, J-P., Brackenbury, R., Rutishauser, U. & Edelman, G.M. (1977). Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* **252**: 6841-6845.

Thiery, J-P., Duband, J-L. & Delouvee, A. (1985). The role of cell adhesion in morphogenetic movements during early embryogenesis. *In the cell in contact*, Edelman, G. & Thiery, J. Eds. New York: Wiley, pp. 169-196.

Timpl, R. & Dziadek, M. (1986). Structure, development and molecular pathology of basement membranes. *Int. Rev. Expl. Pathol.* **29**: 1-112.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.M. & Martin, G.R. (1979). Laminin-a glycoprotein from basement membranes. *J. Biol. Chem.* **254**: 9933-9937.

Todd III, R.F., Arnaout, M.A., Rosin, R.E., Crowley, C.A., Peters, W.A. & Babior, B.M. (1984). Subcellular localization of the large subunit of Mol, a surface glycoprotein associated with neutrophil adhesion. *J. Clin. Invest.* **74**: 1280-1290.

Todd, I. & Gingell, D. (1980). Red blood cell adhesion. I. Determination of the ionic conditions for adhesion to an oil-water interface. *J. Cell Sci.* **41**: 125-133.

Tranzer, J.P. & Baumgartner, H.R. (1967). Filling gaps in the vascular endothelium with blood platelets. *Nature (London)*, **216**: 1126-1128.

Trinkaus, J.P. (1969). Cells into organs. The forces that shape the embryo. Prentice-Hall, Inc. Englewood Cliffs, New Jersey.

Truneh, A., Albert, F., Goldstein, P. & Schmitt-Verhulst, A-M. (1985). Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature (London)*, **313**: 318-320.

Tsien, R.Y., Pozzan, T. & Rink, T.J. (1982). T-cell mitogens cause early changes in cytoplasmic free calcium and membrane potential in lymphocytes. *Nature, (London)*, **295**: 68-71.

Ueda, M.J., Ito, T., Okada, T.S. & Ohnishi, S-I. (1974). A correlation between membrane fluidity and the critical temperature for cell adhesion. *J. Cell. Biol.* **71**: 670-674.

Underwood, P.A. & Bennett, F.A. (1989). A comparison of the biological activities of the cell adhesive proteins vitronectin and fibronectin. *J. Cell Sci.* **93**: 641-649.

Unwin, P.N.T. & Zampighi, G. (1980). Structure of the junction between communicating cells. *Nature (London)*, **283**: 545-549.

Vallee, E., Gougat, J., Navarro, G. & Delahayes, J.F. (1979). Anti-inflammatory and platelet anti-aggregant activity of phospholipase A_2. *J. Pharm. Pharmacol.* **31**: 588-592.

Van Meer, G., Gumbiner, B. & Simons, K. (1986). The tight junction does not allow lipid molecules to diffuse from one epithelial cell to the next. *Nature (London)*, **322**: 639-641.

Verwey, E.J.W. & Overbeek, J.Th.G. (1948). Theory of the stability of lyophobic colloids. Amesterdam: Elsevier.

Vickers, J.D., Kinlough-Rathbone, R.L. & Mustard, J.F. (1984). Changes in the platelet phosphoinositide during the first minute after stimulation of washed rabbit platelets with thrombin. *Biochem. J.* **219**: 25-31.

Vickers, M.G. & Edwards, J.G. (1972). The effect of neuraminidase on the aggregation of BHK 21 cells and BHK 21 cells transformed by polyoma virus. *J.Cell Sci.* **10**: 759-768.

Vigdahl, R.L., Marquis, N.R. & Tavormina, P.A. (1969). Platelet aggregation. II. Adenyl cyclase, prostaglandin E_1 and calcium. *Biochem. Biophys. Res. Commun.* **37**: 409-415.

Volpi, M., Yassin, R., Tao, W., Molski, T.F.P., Naccachi, P.H. & Sha'afi, R.I. (1984). Leukotriene B_4 mobilizes calcium without the breakdown of polyphosphoinositides and the production of phosphatidic acid in rabbit neutrophils. *Proc. Natl. Acad. Sci. (USA)*. **81**: 5966-5969.

Volwerk, J.J., Pieterson, W.A. & De Haas, G.H. (1974). Histidine at the active site of phospholipase A_2. *Biochem.* **13**: 1446-1454.

Warner, A.E., Guthries, S,E. & Gilula, N.B. (1984). Antibodies to gap junctional protein selectively disrupt junctional communication in the early amphibian embryos. *Nature (London)*, **311**: 127-131.

Wayner, E.A. & Carter, W.G. (1987). Identification of multiple cell adhesion receptors for Collagen and fibronectin in human fibrosarcoma cell possessing unique alpha and common beta subunits. *J. Cell Biol.* **105**: 1873-1884.

Webster, R.O., Wysolmerski, R.B. & Lagunoff, D. (1986). Enhancement of human neutrophil adherence to plastic and endothelium by phorbol myristate acetate. Comparison with human C5a. *Am. J. Pathol.* **125**: 369-378.

Weeks, G. (1976). The manipulation of the fatty acid composition of *Dictyostelium discoideum* and its effect on cell differentiation. *Biochim. Biophys. Acta.* **450**: 21-32.

Weiss, H.J. & Turrito, V.T. (1979). Prostacyclin (PGI_2) inhibits platelet adhesion and thrombus formation on subendothelium. *Blood*, **53**: 244-250.

Weiss, L. (1968). Studies on cellular adhesion in tissue culture: X, an experimental and theoretical approach to interaction forces between cells and glass. *Expl. Cell Res.* **53**: 603-614.

Weiss, L. (1973). Neuraminadase, sialic acids, and cell interactions. *J. Natl. Cancer Inst.* **50**: 3-19.

Weiss, L. (1984). Cell adhesion and metastasis. *In matrix and cell differentiation*, Kemp, R.B. & Hinchliffe, J.R. Eds. Alan R. Liss, Inc., New York. pp. 371-386.

Weiss, L. & Harlos, J.P. (1972). Short-term interaction between cell surfaces. *Prog. surface sci.* **1**: 355-405.

Weiss, L. & Chang, M.K. (1973). Some effects of actinomycin D, Cyclohexamide and puromycin on cell adhesion. *J. Cell Sci.* **12**: 655-664.

Weissmann, G., Goldstein, I. & Hoffstein, S. (1976). Prostaglandins and the modulation by cyclic nucleotides of lysosomal enzyme release. *Adv. Prostaglan. Thrombox. Res.* **2**: 803-814.

Weissmann, G., Azaroff, L., Davidson, S. & Dunham., P. (1986). Synergy between phorbol esters, 1-oleyl-2-acetylglycerol, urushiol, and calcium ionophore in eliciting aggregation of marine sponge cells. *Proc. Natl. Acad. Sci.* **83**: 2914-2918.

Weller, P.F., Lee, C.W., Foster, D.W., Corey, E.J., Austen, K.F. & Lewis, R.A. (1983). Generation and metabolism of 5-lipoxygenase pathway leukotrienes by human eosinophils: Predominant production of leukotriene C_4. *Proc. Natl. Acad. Sci. (USA).* **80**: 7626-7630.

Weston, J. (1970). The migration and differentiation of neural crest cells. In *advances in morphogenesis*, **Vol.8**, Aberkrombie, M., Brachet, J., & King, T. Eds. New York, London: Academic Press. pp. 41-114.

White, D.C. & Tucker, A.N. (1969). Phospholipid metabolism during bacterial growth. *J. Lipid Res.* **10**: 220-233.

White, J.R., Naccache, P.H., Molski, T.F.P., Borgeat, P. & Sha'afi, R.J. (1983). Direct demonstration of increased intracellular concentration of free calcium by chemotactic factor. *Biochem. Biophys. Res. Commun.* **113**: 44-50.

Whittle, B.J.R. & Moncada, S. (1983). Pharmacological interactions between prostacyclin and thromboxanes. *Br. Med. Bull.* **39**: 232-238.

Wight, T.N., Raugi, G.J., Mumby, S.M. & Bornstein, P. (1985). light microscopic immunolocalisation of thrombospondin in human tissues. *J. Histochem. Cytochem.* **33**: 295-302.

Wilkins, D.J., Ottewill, R.H. & Bangham, A.D. (1962). On the flocculation of sheep leukocytes. 1. Electrophoretic studies. *J. Theor. Biol.* **2**: 176-191.

Wilkinson, P.C., Lackie, J.M., Haston, W.S. & Islam, L.N. (1988). Effect of phorbol esters on shape and locomotion of human blood lymphocytes. *J. Cell Sci.* **90**: 645-655.

Williams, R.E., Wisnieski, B.J., Rittenhouse, H.G. & Fox, C.F. (1974). Utilization of fatty acid supplements by cultured animal cells. *Biochem.* **13**: 1969-1977.

Willingham, M.C. & Pastan, I. (1975). Cyclic AMP and cell morphology in cultured fibroblasts. *J. Cell Biol.* **67**: 146-159.

Yamada, K. & Olden, K. (1978). Fibronectins: Adhesive glycoproteins of cell surface and blood. *Nature (London)*, **275**: 179-184.

Yamada, K.M. (1983). Cell surface interactions with extracellular materials. *Ann. Rev. Biochem.* **52**: 761-799.

Yamazaki, M., Gomez-Cambronero, J., Durstin, M., Molski, T.F.P., Becker, E.L. & Sha'afi, R.I. (1989). Phorbol 12-myristate 13-acetate inhibits binding of leukotriene B_4 and platelet activating factor and the responses they induce in neutrophils: site of action. *Proc. Natl. Acad. Sci. (USA).* **86**: 5791-5794

Yogeeswaran, G. (1981). Incorporation of a sialo G_{M2} and gangliosides in cell surface of cultured metastatic and nonmetastatic Balb/3T3 cell line: Altered adhesion to substrate *in vitro* and subcutaneous tumor cell take. *J. Natl. Cancer Inst.* **66**: 303-310.

Yoshida-Noro, C., Suzuki, N. & Takeishi, M. (1984). Molecular nature of the calcium dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with monoclonal antibody. *Dev. Biol.* **101**: 19-27.

Zavoico, G.B., Halenda, S.P., Sha´afi, R.I. & Feinstein, M.B. (1985). Phorbol myristate acetate inhibits thrombin-stimulated calcium mobilization and phosphatidyl inositol 4,5-bisphosphate hydrolysis in human platelets. *Proc. Natl. Acad. Sci. (USA)*. **82**: 3859-3862.