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UNIVERSITY OF ALBERTA

BARRIER PROPERTIES OF EPITHELIAL AND ENDOTHELIAL CELLS IN CULTURE: A SERIES OF BIOCHEMICAL AND TRANSPORT STUDIES

BY

CHANGXIN LI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF **DOCTOR OF PHILOSOPHY**

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL, 1990

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Dear Sir/Madame:

I herewith express my consent to the inclusion of our joint work in the above thesis.

Sincerely yours,

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P. K. T. PANG

S. HARVEY

B. R. STEVENSON

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June 25, 1990

DEDICATED WITH DEEP LOVE TO:

MY WIFE QINGLING (LIU);

MY PARENTS;

MY UNCLE AND AUNT; AND

MY PARENTS-IN-LAW

A B S T R A C T

Madin-Darby canine kidney (MDCK) epithelial and bovine aortic endothelial cultures were used to study the barrier properties of the cells in monolayer form. These two types of cells exist in monolayer not only *in vitro*, but also *in vivo*. We have demonstrated that low density lipoprotein (LDL) receptors are present on both apical and basal surfaces of confluent MDCK epithelial monlayer. However, the two groups of receptors function differently. The basal receptors behave very much like typical LDL receptors found in other cell systems. They can be regulated and they in turn can regulate internal cholesterol biosynthesis. The apical receptors, on the other hand, are not sensitive to regulation by LDL but appear to be important in mediating the transcellular transport or transcytosis of LDL. These functionally distinct receptors seem to be biochemically similar as determined by LDL and very low density lipoprotein (VLDL) binding and by immunoblotting with an anti-LDL receptor antibody.

In a second series of studies examining a tight junction-associated protein ZO-1 in endothelial and other types of cell cultures we have shown that the protein is localized immunofluorescently at the cell peripheries where cell-cell contacts are observed in endothelial monolayers. The content of the protein is positively correlated with the monolayer cell density or confluency. The protein is also examined in other types of cells. It is present in MDCK cells, as already been shown, in rat intestinal epithelial cultures (IEC-6), but not in a mouse myeloma

culture. It seems that the protein is exclusively associated with endothelium and epithelium.

Protein kinase C, cyclic AMP, and an uncoupler of oxidative phosphorylation, FCCP, were examined for their role in regulating tight junctions or transepithelial resistance in MDCK cells. Activation of protein kinase C prevented the development of transepithelial resistance in MDCK monolayers. It induced a dramatic decrease in transepithelial resistance in the monolayers which had already generated high resistance levels. The drop in transepithelial resistance by protein kinase C activation was accompanied by a diffusion of the tight junction-associated protein ZO-1 out of cell peripheries, as visualized by immunofluorescence microscopy. In contrast, cyclic AMP has the opposite effect on transepithelial resistance in such monolayers. It makes the tight junctions less permeable. This resistance-increasing effect is observed within 1 to 2 hours following additions of a cyclic AMP analogue and cyclic AMP-elevating agents. Prolonged incubation of the monolayers with these agents caused decreases in transepithelial resistance. The levels came to the original values in about 10 hours and continued to decrease up to 24 hours. This long-term effect is probably related to cell proliferation induced by cyclic AMP. FCCP, an uncoupler of oxidative phosphorylation or a proton gradient depleter, caused MDCK monolayer transepithelial resistance to drop in a few seconds. Once the drug was withdrawn from the incubation, the transepithelial resistance could recover to close to the original levels in less than 2 hours. The decrease in resistance by FCCP was accompanied by a diffusion of the

tight junction-associated protein ZO-1 out of cell peripheries. Transepithelial resistance recovery after FCCP withdrawal was also accompanied by reconcentration of the protein back to the cell peripheries. This points to the dynamic nature of the tight junction structure. The effect of FCCP on the tight junction is probably mediated by intracellular pH changes.

A C K N O W L E D G E M E N T S

The completion of this thesis and all the research work would not have been possible without the supervision and help from those surrounding me. Dr. Mark J. Poznansky impresses me most by being a supervisor who constantly encourages new ideas and always lets them be acted upon, and being a colleague who is easy-going, moderate and understanding. Dr. Bruce R. Stevenson provided the monoclonal antibody anti-ZO-1 and critically reviewed every piece of the work on the protein ZO-1. This thesis would be very different without his generous help. Jennifer Halford helped me to start my laboratory research here. She will be remembered not only as a good tutor but also as a pleasant person. Deanna Moores and Donna Taylor provided most of the cell cultures used. They made their major contributions in forms of labor and technical excellence. I thank Sandra Czekanski for helping with the purchases and my English. I am grateful to Drs. Peter K. T. Pang, Dennis E. Vance, Stephen Harvey, Athony K. Ho, Constance Ho, Wolfgang J. Schneider and Richard A. Murphy for their supervision and help. My wife Qingling (Liu) made this easier by direct participation, continuous encouragements, the tolerance to my long working hours and the dedications as a wife and mother. My gratitude also goes to my son Lennie Benjamin Chouchou for making my scientific life richer and more colorful. Annette Aarbo, Wayne Kwasnisky and Joyce Gatzke are deeply appreciated for their excellent typing work. On the thanks list are also Shirley Willard, Le Luong, Vivian Orozco, Helene Plichta, Dr. Robert Berdan, Dr. David Shiff, Dr. Yair Amit, Guodong Mao, Jie Shan, Michelle Scarth and Koujong Lin for their help and friendship.

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LIST OF ABBREVIATIONS

anti-ZO-1: the monoclonal antibody against the protein ZO-1

ATCC: American Type Culture Collection

ATP: adenosine triphosphate

Bt2cAMP: N^6, 2'-O-dibutyryladenosine 3': 5'-cyclic monophosphate

Bt2cGMP: N^2, 2'-O-dibutyrylguanosine 3': 5'-cyclic monophosphate

cAMP or

cyclic AMP: 3': 5'-cyclic adenosine monophosphate

CURL: compartment for uncoupling of receptor and ligand

DMEM: Dulbelcco's modified Eagle's medium

DMSO: dimethyl sulfoxide

EDTA: disodium ethylene diamine-tetraacetate

EGF: epidermal growth factor

EGTA: ethylene glycol bis-(beta-aminoethyl ether) N, N, N', N'-tetraacetic acid

F-12: medium F-12

FCCP: carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone

FCS: fetal calf serum or fetal bovine serum

GERL: Golgi endoplasmic reticulum lysosome

H7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine

IEC-6: a rat intestinal epithelial cell line

LDL: low density lipoprotein

LLC-PK_1 a pig kidney proximal tubular cell line

MDCK: Madin-Darby canine kidney

MEM: minimum essential medium

PBS:	phosphate buffered saline
PMA:	phorbol 12-myristate 13-acetate
RPMI 1640:	medium RPMI 1640
RMT	receptor-mediated transcytosis
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA:	trichloroacetic acid
TER:	transepithelial electrical resistance
TLC:	thin-layer chromatography
VLDL:	very low density lipoprotein
W7:	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide
ZO-1:	a tight junction protein

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

ABBREVIATIONS

cyclic AMP or cAMP: 3': 5'-cyclic adenosine monophosphate

CURL: compartment for uncoupling of receptor and ligand

FCCP: carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone

GERL: Golgi endoplasmic reticulum lysosome

H7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine

LDL: low density lipoprotein

LLC-PK_1: a pig kidney proximal tubule cell line

MDCK: Madin-Darby canine kidney

TER: transepithelial electrical resistance or transepithelial resistance

RMT: receptor-mediated transcytosis

RT: renal tubule

GENERAL INTRODUCTION

The original intent of this thesis project was to examine the barrier properties of endothelial cells grown in continuous culture. Our laboratory is interested in the use of biological macromolecules as carriers of drugs/enzymes and the notion of site-specific drug or enzyme delivery (Poznansky, 1987). As such it is essential to have a good understanding of how peptides both small and large exit the circulation to arrive at target tissues. Until recently the phenomenon or process was little studied either *in vivo* or *in vitro* and textbooks carried the original teachings of those emphasizing the passive nature of macromolecular permeability of the vascular endothelium (Pappenheimer, et al, 1951, Pappenheimer, 1953). Some of these notions have recently been challenged (King and Johnson, 1985, Milici et al., 1987, Simionescu et al., 1987). The ability to culture and study endothelial cells in appropriate forms emerged in the 1970's and reports began to appear on the barrier properties of the endothelial monolayer. This was to have been the principal subject matter of this thesis. We sought to examine the mechanism or mode by which macromolecules are transported across the cultured monolayer in endothelium. Unfortunately, we were not able to produce stable endothelial monolayers with low background permeabilities to carefully monitor specific peptide transport.

In attempting to refine this experimental protocol we began also to work on a line of epithelial cells (MDCK) which represent a more established cell system and which are known to generate low permeability monolayers in culture. The data examined in this thesis refer for the greater part to the experiments carried out on the MDCK cells, and the

nature of its barrier properties. Several questions were subsequently asked.

1. How do macromolecules, like the low density lipoprotein (LDL) particle, traverse the epithelial monolayer? At the same time, in order to further understand the interactions of LDL with the MDCK monolayer we examined the role of LDL in the control of cholesterol biosynthesis in MDCK cells and the asymmetry of LDL receptor and LDL metabolism in the cells (basal and apical cell surfaces).

2. What is the role of the tight junction protein ZO-1 in the integrity of endothelial and epithelial monolayers? Is this protein present only once the tight junctions are formed?

3. What factors control tight junction permeability? Among the factors we examined are second messengers protein kinase C and cAMP, proton gradient depleter FCCP, and epidermal growth factor.

While the original intent of the program was not realized we have gained considerable information about the barrier properties of epithelium with some information from endothelial cell in cultures, especially in regard to the tight junction. This thesis is presented in paper format describing five discrete efforts in understanding the nature of this barrier.

THE CELL SYSTEMS

This section is designed to discuss the major cell systems used in the thesis.

EPITHELIUM AND ENDOTHELIUM

Epithelial cells lining the cavities of natural lumens (intestinal, kidney tubular, etc.) are derived embryologically from endoderm. These cells are joined together to form cell layers separating two different environments. Such monolayers have two functions: as a restrictive barrier by virtue of the formation of tight junctions between cells and as a communicating barrier between compartments as indicated by the presence of varied transport mechanisms and in some instances the lack of complete sealing of the intercellular spaces (Alberts et al., 1983). Some types of epithelial cells such as Madin-Darby canine kidney (MDCK) cells in confluent monolayer have well-developed tight junctions and generate a high level of transepithelial electrical resistance (TER), which makes them ideal to study the properties of barrier-forming cells (epithelium, endothelium and mesothelium, etc.). Adapted to the two different environments, an epithelial cell monolayer either *in vivo* or *in vitro* generates two distinct surfaces: an apical one facing the lumen in the body (e.g. plasma or urine) or medium in culture and a basal (or basolateral) one facing sub-epithelial tissues *in vivo* or the support (dish or membrane filter) in culture. Differences in these two surfaces with respect to composition, topography and function, etc. are referred to as the asymmetric or polar nature of these cells.

Endothelial cells share many of the properties of epithelial tissue even though they are derived embryologically from a different germ layer, the mesoderm. These cells form the linings between two different internal (instead of internal and external in case of epithelial cells) body

environments, i.e. tissues and blood or lymph. The linings function as a selectively permeable barrier. These cells in monolayer have been demonstrated to show some asymmetry (Muller and Gimbrone, Jr., 1986, Jaffe et al., 1987). Tight junctions have been recognized in some types of endothelial cells (Schneeberger and Lynch, 1984), especially the brain capillary endothelium (Goldstein and Bentz, 1986, Bradbury, 1984). In addition, an active transcytosis process has been shown to exist in endothelial cells (Simionescu et al., 1987, King and Johnson, 1985, Milici et al., 1987).

In summary, epithelial and endothelial cells share many properties including monolayer-formation (both in *in vivo* and in culture), tight junction development and monolayer asymmetry.

MADIN-DARBY CANINE KIDNEY CELL

Madin-Darby canine kidney or MDCK cells represent a cell line originally derived from the tubules of a normal dog (Madin and Darby, 1958, Gaush et al., 1966). The origin of the cell is somewhat controversial, but it is generally agreed that the cell line has an origin of distal tubules since the cells were found to lack detectable levels of several proximal tubular markers such as trehalase, maltase, Na^+-dependent D-glucose uptake, and Na^+-dependent *p*-aminohippurate uptake (Gaugh et al., 1966). The cell line has been used and characterized extensively (Cereijido et al., 1978, Gaugh et al., 1966, McRoberts et al., 1981, Rindler et al., 1979, Rabito et al., 1978, Saier, Jr., 1981). The cell line retains the differentiated properties of the cells from which it was derived (Rindler, 1979). The cell line

behaves as typical epithelium including tight junction formation (Cereijido et al., 1978, Rabito et al., 1978, Gonzalez-Mariscal et al., 1985, Cereijido et al., 1980) and monolayer asymmetry (Cereijido et al., 1978, Herzlinger and Ojakian, 1984, Balcarova-Stander et al., 1984). The cell line has been extensively used for studying these functions. MDCK monolayers generate largely variable TER depending on the culture condition, passage number and type of clone. The reported TER values are from one extreme of more than 4000 ohms.cm^2 to the other of less than 100 ohms.cm^2 (Barker and Simmons, 1981, Richardson et al., 1981, Hanson et al., 1986). The cells we used can generate TER between these two extremes. The MDCK cell has a very low nutritional requirement. For example, it can survive and grow in serum-free minimum essential medium, even though richer media or supplements are often used to speed up the growth rate, or to slow down the degenerative changes (our experience and observations).

EPITHELIAL ASYMMETRY

Simons and Fuller (1985) have presented a comprehensive review of the area of epithelial asymmetry. Most types of epithelia (transporting epithelia such as those of the renal tubule, absorptive epithelia such as those of the intestine and secretory epithelia such as hepatocytes, etc.) are organized in sheets or monolayers both *in vivo* and *in vitro* by the formation of tight junctions intercellularly. The plasma membrane of each cell in monolayer is divided into two distinctively different surfaces: apical and basal, which confers on the monolayer the property of asymmetry.

Compositional asymmetry refers to the localization of molecules (lipids, proteins or carbohydrates) preferentially or specifically on one surface rather than the other. It has been shown that viruses budding from apical and basal membranes have different lipid compositions in MDCK cells (van Meer and Simons, 1982) and in Madin-Darby bovine kidney cells (Rothman et al., 1976). The apical membrane from mouse intestinal epithelium is enriched in glycolipids and cholesterol compared to the basal equivalent (Kawai et al., 1974). The asymmetry of membrane proteins, however, are much better understood than that of the lipids. A large number of proteins, mostly membrane proteins or membrane-associated proteins have been mapped specifically to one of the two surfaces. Usually such surface-specific proteins are specific for all types of epithelia with a few exceptions. Some of these proteins, especially enzymes are used as surface markers. Simons & Fuller (1985) have presented an excellent review summarizing asymmetric properties of a variety of such proteins in several types of epithelia. Functional asymmetry is exemplified by unidirectional transport of small electrolytes and nonelectrolytes, and vectorial transcytosis of selected proteins (see bellow) .

Table I-1 shows a few selective asymmetric proteins associated with MDCK culture and renal tubular cells.

Endothelial cells show similar asymmetry. Some common epithelial surface markers such as alkaline phosphatase (apical marker) and Na^+, K^+-ATPase (basal marker) also apply to the endothelium (Jaffe et al., 1987). By the technique of surface-selective iodination,

Muller and Gimbrone (1986) revealed at least a dozen different plasma membrane proteins which are localized specifically to either apical or basal cell surface of cultured vascular endothelial cells. As shown in epithelial cells (Gottlieb et al., 1986), budding of viruses from endothelial cells in monolayer is asymmetric (Lombardi et al., 1985). Angiotensin-converting enzyme is not only a marker for the endothelial cell type but also a domain marker for the apical surface (Nakache et al., 1986).

The morphogenesis of asymmetric epithelial cell sheets has recently been reviewed by Roudriguez-Boulan and Nelson (1989). The generation of polarized epithelial cells start from the very beginning of embryogenesis. The earliest stage at which cell polarity is established is when the trophectoderm is formed from the outer blastomeres. Trophectoderm is a group of polarized epithelial cells surrounding a fluid-filled lumen, the blastocoel, and the inner cell mass. Most specialized epithelia in many organs or tissues, for example kidney tubular epithelium, are developed from the branching or growing of epithelial buds (Ekblom et al., 1986). The generation of cell polarity appears to be initiated by both cell-cell contact and by cell-substratum contact. Cell-cell contact results in the formation of junctional complexes, including the tight junctions. Cell-substratum contact induces cell polarity without the requirement for actual cell-cell interaction (Vega-Salas et al., 1987). The formation of cell polarity is probably a direct consequence of a sorting mechanism by the Golgi complex and by other organelles (Griffiths and Simons, 1986). Besides the tight junction, membrane-specific anchorage of sorted molecules (Brown et al., 1989) and cytoplasmic anchoring molecules such as

Table I-1 Asymmetric protein markers in epithelial cells

Protein	Cell Type	Surface mainly associated	References
Transferrin receptors	MDCK	Basal	Fuller and Simons, 1986
Influenza hemagglutinin	MDCK	Apical	Pfeiffer et al., 1985
Vesicular stomatitis Virus G protein	MDCK	Basal	Roman et al., 1988
Na^+, K^+ -ATPase	MDCK	Basal	Louvard, 1980
	RT	Basal	Shaver and Stirling, 1978
Aminopeptidase N	MDCK	Apical	Louvard, 1980
	RT	Apical	Kerjaschki et al., 1984
Alkaline phosphatase	RT	Apical	Gomori, 1941
	$LLC\text{-}PK_1$	Apical	Rabito et al., 1984
Gamma-glutamyl transpeptidase	$LLC\text{-}PK_1$	Apical	Rabito et al., 1984
Mg^{++} -ATPase	RT	Apical	George and Kenny, 1973
ATP-dependent Ca^{++} uptake	RT	Basal	Gmaj et al., 1979
Adenylate cyclase	RT	Basal	Schwartz et al., 1974

RT = renal tubule

$LLC\text{-}PK_1$ = a cell line from pig kidney proximal tubule.

ankyrin and spectrin (Nelson and Veshnock, 1986, Nelson and Harmmerton, 1989) have been thought to play a role in maintaining cell polarity.

TRANSCYTOSIS

TRANSPORT ACROSS MONOLAYER

There are two routes through which molecules can cross a cell monolayer: one is that through the interior of the cells (cellular or transcellular pathway) and the other is through the spaces between the cells or through tight junctions (paracellular pathway). The latter will be discussed later in detail in the section on the Tight Junction.

In order for a molecule to go through the cellular pathway, it has to pass two plasma membrane bilayers and transverse the cytoplasm. Cellular transport can be done by one or more of the following mechanisms. (1) Simple diffusion: This mechanism follows Fick's first law (Kotyk et al., 1988). Small molecules such as water, urea, O_2 and lipid soluble molecules such as cholesterol, bile acids and lipid-soluble vitamins are likely transported across cell monolayers by this mechanism. (2) Carrier-mediated transport: This type of transport can be either energy-dependent (active transport) or energy-independent (facilitated transport). Good examples are the transport of nutrients (glucose and amino acids, etc.) and metal ions across intestinal epithelium (absorption) and kidney tubular cells (re-absorption or secretion). Specific transporters or carriers are the basis for such transport. (3) Transcytosis (Simionescu et al., 1987): Macromolecules (proteins) are mainly transported across a cell by this mechanism because of their large size. Transcytosis can be either receptor-mediated or without the

involvement of a specific receptor. In the latter case, a phenomenon similar to fluid-phase pinocytosis might be involved. Receptor-mediated transcytosis (RMT) will be discussed in detail in the next section.

RECEPTOR-MEDIATED TRANSCYTOSIS

The only possible mechanisms for macromolecules such as proteins to cross epithelial (or endothelial) barriers are by transcytosis or a leak pathway through intercellular spaces. Transcytosis is a process associated with membrane invagination or endocytosis, vesicle shuttling and exocytosis (Simionescu et al., 1987, Milici et al., 1987, Poznansky and Juliano, 1984). If it were shown that the process is specific and involves a receptor, then the process might be termed receptor-mediated transcytosis. In epithelial cells, several proteins (Table I-2) have been demonstrated to cross the monolayer through transcytosis and in a vectorial way. The mechanism for the transport to be vectorial is not fully understood.

Vectorial tran cytosis is probably accomplished partly by a specific sorting mechanism of the cell. Sorting deals with both the products synthesized by the cell and the molecules imported. These molecules are delivered to the specific surface by such a sorting mechanism. The structural basis for the sorting of newly synthesized molecules is thought to be Golgi complex or Golgi endoplasmic reticulum lysosomes (GERL) (Griffiths and Simons, 1986), and for the sorting of imported ones (either transcytosis or receptor-mediated endocytosis) to be endosomes (Helenius et al., 1983) with possible involvement of GERL, CURL (compartment for uncoupling of receptor and ligand), or other structures. Various pathways

Table I-2 Proteins undergoing vectorial transcytosis in epithelia

Protein	Cell Type	Direction	Mode	References
Epidermal growth factor	MDCK	B --> A	RMT	Maratos-Flier, 1987
Thyroglobulin	Pig thyroid gland	B --> A	RMT	Herzog, 1983
G protein of vesicular stomatitis virus	MDCK	A --> B		Personen et al.,1984
IgG	Rat enterocyte	A --> B	RMT	Abrahamson & Rodewald, 1981
Asialoglycoprotein	Hepatocyte	B --> A	RMT	Schiff et al., 1984
IgA	MDCK	B --> A	RMT	Mostov & Deitcher, 1986
Transferrin receptor	MDCK	B --> A	RMT	Fuller & Simons, 1986

A --> B = from apical to basal sides

B --> A = from basal to apical sides

RMT = receptor-mediated transcytosis

of receptor-mediated transcytosis or endocytosis share one common feature: the receptors move to the coated pits or coated vesicles. Ligands may bind to the receptors before or after the event. Ligand-receptor complexes finally end up in the endosomal structure (Goldstein et al., 1985). The contents of the endosomes may be delivered to the lysosomes for degradation, but in some instances may escape the lysosomes, for example in the transcytosis of immunoglobins across epithelial monolayers (Solari and Kraehenbuhl, 1984). Microtubules or microfilaments have been suggested to play a role in directing the movement of the transcytosed molecules or particles (Danielsen et al., 1983, Rindler et al., 1984). Where the

sorting signals come from is largely a mystery.

The transcytosis of proteins across endothelial cells is also now well documented. Albumin (Milici et al., 1987), insulin (King and Johnson, et al., 1985), transferrin (Fishman et al., 1987), LDL (Hashida et al., 1986) and ceruplasmin (Irie and Tavassoli, 1986) have been demonstrated to be transported across endothelial cell monolayers by receptor-mediated transcytosis or by simple transcytosis.

LOW DENSITY LIPOPROTEIN: METABOLISM VS TRANSCYTOSIS

The metabolism of low density lipoprotein (LDL) has been well studied. J. L. Goldstein and M. S. Brown were recognized for their original work on LDL and cholesterol metabolism by the 1985 Nobel prize in Physiology and Medicine (Brown and Goldstein, 1986). LDL enters the cell by a process called receptor-mediated endocytosis using the LDL receptor (Goldstein et al., 1985). The LDL receptor is a membrane protein, with a cytoplasmic C-terminal and an extracellular N-terminal (Schneider, 1989). This general structure is shared by many receptors mediating endocytosis: IgA/IgM receptor (Mostov et al., 1984), epidermal growth factor receptor (Ullrich et al., 1984), insulin receptor (Ebina et al., 1985), transferrin receptor (McClelland et al., 1984) and the asialoglycoprotein receptor (Drickamer et al., 1984), etc.. The metabolic pathway for LDL in fibroblasts and other cells and the role of LDL as a nutrient has now been well defined (Goldstein and Brown, 1986). LDL binds to its receptor or cell surface. The bound LDL is internalized through invagination of the involved plasma membrane in a coated pit and is then delivered to lysomomes through membrane vesicles or endosomes. The LDL receptor will

dissociate from LDL and recycle back to plasma membrane through a structure called CURL (compartment for uncoupling of receptor and ligand) for further LDL transport. The LDL is then degraded in lysosomes. The released cholesterol, upon reaching the endoplasmic reticulum, produces three consequences in terms of control of internal cholesterol biosythesis: inhibiting the rate-limiting enzyme of cholesterol synthesis 3-hydroxy-3-methylglutaryl coenzyme A reductase (Balasubramaniam et al., 1977); increasing the synthesis of the storage form of cholesterol, cholesteryl esters, by increasing the activity of acyl CoA: cholesterol acyltransferase; decreasing the further import of LDL by decreasing the synthesis of new LDL receptors. This pathway is based on the assumption that all the LDL is degraded by cells.

It has been shown that the internalized LDL can come back on to its surface without degradation in human fibroblasts (Aulinskas et al., 1985, Greenspan and St. Clair, 1982). In other words, the internalized LDL can by-pass the lysosomes and be externalized. This process is called retro-endocytosis. If LDL is endocytosed from one side of the cell and retro-endocytosed from the other side of it, then the combined process is the aforementioned transcytosis or, in fact, receptor-mediated transcytosis. LDL has been shown to be transcytosed across the endothelial monolayer (Hashida et al., 1986, Territo et al., 1984). By the same token LDL may be able to cross epithelial monolayers by transcytosis. This is the subject matter of the first paper (Chapter II).

Even though the dog is not a popular species used in the study of LDL and cholesterol biosynthesis, the LDL receptor has been demonstrated in

dog liver (Mahley et al., 1981, Wade et al., 1986).

TIGHT JUNCTION

GENERAL REVIEW

Farquhar and Palade (1963), in describing the morphology of junctional complexes in a variety of epithelial cells, distinguished three components: (1) a discontinuous *zonula adhaerens*, (2) a distal button-like *macula adhaerens* or desmosome, (3) a *zonula occludens* or tight junction. The tight junction is one of the most unique properties associated with epithelial and endothelial cells (Schneeberger and Lynch, 1984). It is usually located near the apical side between the neighboring cells and forms the division between apical and basal (or basolateral) surface domains (Sabatini et al., 1983). Tight junction as a barrier was originally demonstrated by using protein and ion tracers, for example horseradish peroxidase (Reese and Karnovsky, 1967) and lanthanum (Dym and Fawcett, 1970). Tight junction was shown to have limited permeability to these tracers. It is due to the formation of tight junctions that monolayers of epithelia and some types of endothelia can generate high TER. The terminology "tight" junction is sometimes misleading, since these junctions vary considerably in their permeability properties. In fact, some are quite permeable. "Occluding junction" would be a more accurate description.

Tight junctions restrict the passing of not only macromolecules (proteins), but also small ions. The latter property is the basis of using TER measurements to indicate the tightness of the tight junctions (Cereijido et al., 1978, Cereijido et al., 1980, Gonzalez-Mariscal et al.,

1985). Since the resistance across plasma membrane bilayers is so high compared to that generated through tight junctions or intercellular spaces, the measured resistance across a monolayer is close to the value for the tight junctions. This fact is explained in the following relationships:

$$\frac{1}{R_T} = \frac{1}{R_M} + \frac{1}{R_I}$$

where R_T = total resistance across cell monolayer,

R_M = resistance by plasma membrane bilayers,

R_I = resistance attributable to intercellular spaces.

When R_M = several orders of magnitude of R_I,

$R_T = R_I$ roughly.

STRUCTURE, COMPOSITION AND FUNCTIONS

This section is written with extensive reference to the articles of Schneeberger and Lynch (1984), Stevenson et al. (1988a), Gumbiner (1987) and Madara (1988, 1989).

The tight junction is formed by a belt structure around one pole of cells in monolayer. It has been postulated to be the site where the neighboring two plasma membranes are "fused" with each other although the studies examining the mobility of lipid molecules in this region suggests there is no continuity of the lipid bilayers (van Meer et al., 1986). Under freeze-fracture electron microscope (EM), the tight junction appears as a group of continuous anastomosing intramembrane strands or fibrils in the P-face (the outside of cytoplasmic leaflet) with complementary grooves

in the E-face (inside of extracytoplasmic leaflet). The number of the strands has been shown to be consistent with the value of TER in intestinal epithelium (Claude, 1978) even with exceptions. The tight junction is probably represented by a common universal structure among all the epithelial cells, since cells from different animal origins, when co-cultured in monolayers, can make sealed tight junctions between them (Gonzalez-Mariscal et al., 1989).

The structural elements which produce the tight junction have not been clearly elucidated. Some authors have proposed that the tight junction is the consequence of membrane lipid fusion (Kachar and Reese, 1982, Kachar and Pinto da Silva, 1981). The evidence for this model was mainly derived from the analysis of freeze fracture replicas of lipid vesicles, composed solely of various combinations of lipids. Tight junction-like intramembrane particles were observed in such cases. The prevailing opinions of tight junction composition nowadays favor the models which are based on the involvement of proteins (van Meer and Simons, 1982, Stevenson et al., 1988a, Madara, 1989). The evidences for protein models come from the observations that cell-cell contact or tight junctions can be disrupted by the treatment with trypsin, a proteolytic enzyme, and that the mRNA sythesis inhibitor actinomycin D can prevent the formation of tight junctions when added during tight junction formation (Griepp, et al, 1983). However, once the tight junctions have formed neither actinomycin D nor the protein synthesis inhibitor cycloheximide has an effect on them (Griepp et al., 1983), suggesting that the protein or proteins participating in the formation of tight junctions are stable.

Identification of the tight junction-associated protein ZO-1 by Stevenson and co-workers (Stevenson et al., 1986, Anderson et al., 1988) represented a milestone in the biochemical characterization of tight junctions. The protein is localized to the vicinity of the cytoplasmic side of the tight junctions. The protein has been described in a variety of epithelial tissues or cultures from several species including MDCK cells (Stevenson et al., 1986, Stevenson et al., 1988a, Anderson et al., 1988, Stevenson et al., 1988b, Siliciano and Goodenough, 1988), human intestinal line Caco-2 cells (Anderson et al., 1989a), mouse colon, kidney, testis (Stevenson et al., 1986) and liver (Anderson et al., 1988), mouse embryo (Fleming et al., 1989), rat liver (Stevenson et al., 1986, Anderson et al., 1989b), and mouse kidney and testis arteriolar endothelial cells (Stevenson et al., 1986). Another newly identified protein associated with tight junctions in avian and mammalian epithelia is cingulin (Citi et al., 1988, Citi et al., 1989), a protein resembling ZO-1 in many aspects. Actin microfilaments have also been shown to have intimate association with the tight junction (Madara, 1987). Despite all of these progresses in characterizing the cytoplasmic components of the tight junction, the molecular identity of the integral membrane elements responsible for the extracellular occlusion have yet to be identified.

The structure of the tight junctions in endothelial tissue under transmission EM appears similar to that in epithelial cells (Albelda et al., 1988, Rutten et al., 1987). However, the composition of the structure is much less well understood, although the protein ZO-1 has been shown to be associated with blood vessel endothelium in the sections of whole tissue (Stevenson et al., 1986).

The functions of the tight junction are primarily twofold: (1) to maintain cellular polarity; (2) to act as a permeability barrier. It has been shown that lipids on one surface domain can not pass the tight junctions to reach the other domain (van Meer and Simons, 1986, Dragsten et al., 1981), and that the tight junction formation precedes the polarization of some proteins such as alkaline phosphatase and gamma-glutamyl transpeptidase (apical enzymes) in renal tubular LLC-Pk_1 cell monolayer (Rabito et al., 1984). The barrier function will be discussed in more detail next.

THE TIGHT JUNCTION: A BARRIER? A GATE?

The transport of molecules across epithelial or endothelial monolayers may occur by one of two pathways: through the interior of the cells and/or through intercellular spaces (Poznansky and Juliano, 1984). The latter is termed the paracellular pathway. The contribution of the paracellular transport to the total transport of molecules across a monolayer should be inversely related to the monolayer TER. In epithelial cells where the tight junctions are clearly well developed, paracellular transport is low. In this sense, the tight junctions function more as a barrier. A barrier is essential to maintain the internal milieu of the organism under chemical and electrical gradients. Disturbance of the tight junction can cause serious diseases in the whole body. For example, renal tubular intoxication (from drugs, ischemia, etc.) will make the tight junctions more permeable and cause the kidney to lose the ability to concentrate urine.

On the other hand, tight junctions may not function as absolute

permeability barriers. They may allow some permeability and in this regard, the term "gate" may be more proper to describe the structure. The gate can be characterized by three different parameters: (1) permeability; (2) permselectivity; and (3) hydraulic conductivity (Schneeberger and Lynch, 1984). Permeability represents the reciprocal of TER. Epithelia from different tissues or from different species generate TER which varies tremendously, ranging from a few ohms.cm^2 in rat proximal convoluted tubule (Boulpaep and Seely, 1971) to several thousand ohms.cm^2 in toad urinary bladder (Civan and Frazier, 1968). Even the same type of epithelium can give different TER when cultured under different conditions. A good example is the MDCK cell line, as discussed previously. Permselectivity of the tight junction is the ability to discriminate between a series of cations and anions (Powell, 1981). Ions with different charge and different ionic potency have different permeability through the tight junction. In fetal lung, for instance, the permeability sequence of the pulmonary epithelium from high to low is Na^+, K^+, Rb^+, Li^+, Cs^+, which is in the same order as that in Eisenman's sequence VIII for alkali metals (Olver and Strang, 1974). In other words, the larger the ionic potency of the ion, the smaller the permeability through the tight junction for cations. These authors proposed that the paracellular channels are covered with fixed negative charges on their walls.

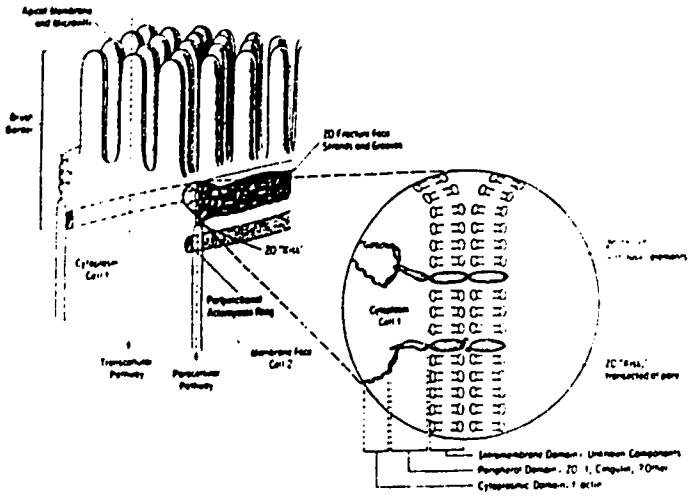
The paracellular pathway in most endothelial cell types may represent a more important pathway than in epithelial cells in terms of contributing to the total transport. For example, bovine aortic endothelial monolayer generates much lower transcellular electrical resistance than MDCK

monolayer (Rutten et al., 1987). It makes it much more difficult to examine the transcellular transport of molecules, especially by using cultured cells. This is one of the reasons why we used epithelium, instead of endothelium, in studying the transport and other properties of cell monolayers even though we had a major interest in the endothelium. If the paracellular pathway is indeed the dominant route through which the macromolecules in circulation cross the blood vessel endothelial layer into the extracirculatory tissues, then the transcellular pathway may not be needed or only play a minor role in the cell type. This has still to be elucidated.

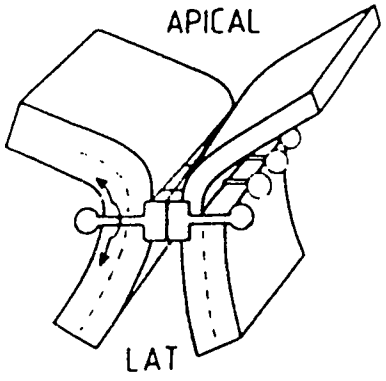
MODELS OF THE TIGHT JUNCTIONS: MADARA, VAN MEER AND SIMONS, STEVENSON AND CO-WORKERS

The few major models of the tight junction discussed here are those based on the hypothesis that protein is the major constituent. Fig. I-1 shows three models proposed by Madara (1989) (upper), van Meer and Simons (1986) (middle), and Stevenson and co-workers (1988a) (lower). In the van Meer and Simon model, a specific transmembrane protein with bulky extracellular portion would pack very densely into a linear array. The protein on one half of the tight junction would have very close contact with that on the other half. Such arrangement would form a permeability barrier to solutes in the extracellular fluid. This model could explain the fact that lipid molecules on the outer leaflet of the membrane bilayer can't pass through the tight junction (van Meer and Simons, 1986). Lipids on the inner leaflet would be free to pass through this structure, since the model proposed that the cytoplasmic potion of the protein would be less

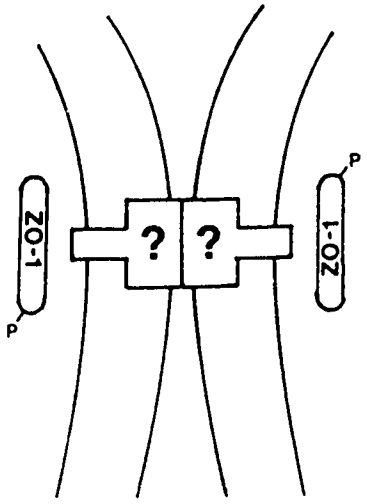
Fig. I-1 Models of tight junction



Madara



van Meer and Simons



Stevenson and co-workers

bulky and less densely packed. In the Madara model, the strands or fibrils seen under freeze-fracture EM would represent sites where intramembrane proteins on the opposing halves of the tight junction "kiss" each other (tight junction "kiss"). The "kiss" could be close enough to form a barrier, or sufficiently far apart to form a pore or channel. The intramembrane protein would be linked to peripheral proteins like ZO-1 and cingulin (Citi et al., 1988), etc. and in turn be linked to the cytoskeleton. This model would explain the influence of the cytoskeleton on the tight junction (Madara et al., 1987, Madara et al., 1988, Madara, 1987). The model by Stevenson et al. (Fig. I-1, lower) is similar to the two models described above, except that the protein ZO-1 would occupy a distinct position. ZO-1 would be in the vicinity of the tight junction formed by paired transmembrane proteins. However, the linkage between ZO-1 and the transmembrane protein is not proposed. All these models are common in one thing: a intramembrane or transmembrane protein is involved, whose chemical identity is not known.

THE TIGHT JUNCTION: A DYNAMIC, REGULATED STRUCTURE

The tight junction is a dynamic structure. Evidence suggests that it must assemble or disassemble either partially or completely during many developmental and physiological processes such as transepithelial migration of neutrophils in inflammation (Milks et al., 1986), migration of maturing spermocytes across the seminiferous tubular epithelium during spermatogenesis (Gilula et al., 1976) and wound healing, etc..

Electrophysiological experiments have shown that a functional tight junction can form very rapidly. After one single epithelial cell of the

Necturus gallbladder was killed, neighboring cells spread to fill the wound in as little as 15 minutes. Within the next 15 minutes new functional tight junctions formed between the cells (Hudspeth, 1982). Martinez-Palomo et al. (1980) showed that the tight junctions can be dissociated in minutes by the depletion of extracellular Ca^{++} and that the dissociated tight junctions could reform within an hour. The latter process is independent of protein synthesis. This evidence suggests that tight junction precursor molecules are readily available in the epithelial (or endothelial) cells. In other words, there is storage or abundance of specific tight junction molecules in the cells that are available to form functional tight junctions in a short period of time.

The mechanism by which Ca^{++} participates in maintaining the integrity of tight junctions is not fully understood. The tight junction contact itself seems not to be dependent on Ca^{++} levels, since treatment of a tight junction-enriched liver membrane preparation with Ca^{++} chelators failed to dissociate the tight junctions (Stevenson and Goodenough, 1984). Other junctional elements may be involved. *Zonula adherens* (Volberg et al., 1986), desmosomes (Watt et al., 1984), uvomorulin or L-CAM (Gumbiner and Simons, 1986, Gumbiner and Simons, 1987) are sensitive to extracellular Ca^{++} depletion. The association of actin filaments with the junctional complex inside the cell also seems to be disrupted by low Ca^{++} levels extracellularly (Volberg et al., 1986).

Intracellular second messengers have been shown to affect tight junctions. Duffey et al. (1981) demonstrated that cyclic AMP analogues

could increase TER in *Necturus* gall bladder epithelium. The TER increase is due to a decreased ion flow through a paracellular pathway. The resistance increase appears to be accompanied by a parallel increase in the thickness of the tight junction belt. In addition to extracellular Ca^{++} affecting tight junction, exposure of *Necturus* gall bladder epithelium to calcium ionophore produces a substantial TER increase following a transient TER drop with concomitant increase in tight junction strands or fibrils (Palant et al., 1983). Protein kinase C activation is also known to increase paracellular ion permeability (Ojakian, 1981).

The regulation of tight junction permeability may have important physiological significance. Madara and Pappenheimer (1987) demonstrated that intestinal epithelial tight junctions or paracellular transport increases in response to increased concentrations of glucose in the intestinal lumen. This represents a compensating mechanism when the glucose transporters are saturated. Glucose-induced increase in paracellular glucose transport is energy-mediated. It is accompanied by structural deformation of the tight junctions and condensation of the perijunctional actomyosin ring. To what extent this paracellular pathway contributes to nutrient absorption from gut or nutrient salvage from kidney tubule is not known although this has been postulated as the physiological role of this property.

It is not clear how intracellular second messengers and glucose trigger the alteration in tight junction structure. The cytoskeleton may be involved. In MDCK cell, manipulation of the cytoskeleton by pharmacologic means results in altered tight junction structure and permeability

(Martinez-Palomo et al., 1980). In intestinal epithelium, tight junction structure is altered when tension is applied laterally by mechanical means (Pitelka et al., 1983) and tight junction permeability is increased when the cells are exposed to cytochalasin D, which specifically interacts with cytoskeleton in a not fully understood manner (Madara et al., 1986). According to Madara (1988), the cytoskeleton may link to the tight junction through the newly identified ZO-1 protein.

CYCLIC AMP, PROTEIN KINASE C AND FCCP

Cyclic AMP (cAMP) is one of the most important second messenger systems. It is probably present in all mammalian cell types. It functions as a messenger translating a variety of receptor stimuli into common responses. Receptor-ligand interactions can stimulate GTP-binding protein (or G-protein) to bind GTP to be activated. The protein also possesses GTPase activity which will hydrolyse GTP to GDP to return itself to an inactive form. Cholera toxin is an enterotoxin found in *V. Cholerae*. It causes sustained activation of the GTP-binding protein by inhibiting GTPase activity (Moss and Vaughan, 1988). Activated GTP-binding protein in turn activates adenylate cyclase which catalyses the transformation of ATP to cyclic AMP. Forskolin activates adenylate cyclase directly without the involvement of the receptor and GTP-binding protein (Seamon, 1981). Cyclic AMP induces cell responses by activating cyclic AMP-dependent kinase and other coupling enzymes. Cyclic AMP itself has a short half life. It is rapidly metabolized by phosphodiesterase to 5'-AMP and loses activity. Protein kinase C is a widely distributed enzyme. Physiologically it is activated through the second messengers following the hydrolysis of

membrane-bound phosphotidylinositol 4,5-biphosphate. Inositol triphosphate is a water soluble product which increases free cytosolic Ca^{++}. Diacylglycerol (Berridge and Irvine, 1984)), another hydrolytic product, activates a Ca^{++}- and phospholipid-dependent kinase: protein kinase C. Phorbol esters which are known as tumor-promoters can activate the enzyme directly (van Duuren, 1969).

Some inhibitors of protein kinases are available although not very specific. For example, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) is most potent and most selective in inhibiting protein kinase C while also being inhibitory on cyclic AMP-dependent protein kinase, cyclic GMP-dependent kinase, myosin light chain kinase (Hidaka et al., 1984, Saitoh et al., 1987), etc..

Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) is an uncoupler of oxidative phosphorylation. It is a lipid-soluble compound. It functions as a carrier for hydrogen ion to cross membrane bilayers. According to electrochemical proton gradient theory for ATP generation, hydrogen ions are driven out of mitochondial membranes by reducing powers (for example, NADH, FADH, etc.) to form a proton gradient, and ATP is generated by ATPase driven by the back flow of the protons down the gradient. When FCCP is present, the proton gradient will dissipate and ATP generation will be prevented. FCCP causes intracellular pH changes due to the effect on the transmembrane proton gradient.

In summary, epithelial and endothelial cells form monolayer both *in vivo* and in culture. The monolayer functions both as a restrictive barrier due to the tight junction formation and a communicating barrier

due to the cellular and paracellular transport mechanisms. The monolayer shows asymmetry in terms of the difference of the two surfaces.

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CHAPTER II

LDL RECEPTORS ON EPITHELIAL CELL (MDCK) MONOLAYERS: ASYMMETRIC DISTRIBUTION AND ROLE IN CHOLESTEROL SYNTHESIS AND LDL TRANSPORT

Abbreviations used:

LDL Low density lipoprotein

VLDL Very low density lipoprotein

LPDS Lipoprotein deficient serum

FCS Bovine serum

ATCC American Type Culture Collection

MDCK Madin-Darby canine kidney

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INTRODUCTION

Madin-Darby canine kidney (MDCK) cells are cultured renal epithelial cells which exhibit a high degree of cellular polarity when grown on a variety of different substrates (2). The cells are derived from the kidney tubular cells which have two distinct surface domains in monolayer form: the apical surface facing the tubular lumen and the basal (or basolateral) surface facing the interstitial fluid, tissues and blood. Similar polarity can be established in tissue culture by growing cells on a porous support system coated with extracellular matrix (3, 4). Polarity is reflected both structurally and functionally (2). Proteins localized on particular surface domains are used as specific surface markers. For instance, Na^+, K^+ ATPase (5, 6) is a domain marker for the basal surface whereas aminopeptidase N (5) is an apical surface marker in MDCK cell monolayers. Similar surface domain localization of other cell surface proteins has been demonstrated both *in vitro* and *in vivo* for several epithelial cell types. A number of cell surface markers have been identified (see ref. 2 for a comprehensive review).

Recent publications have described models for the morphogenesis of these polarized cells (7) and demonstrated important differences in protein composition of the apical and basal plasma membrane domains of MDCK cells (8). Functional polarity of MDCK cells is exemplified not only by the transport of small ions and nonelectrolytes but also by transcytosis, a process in which proteins can be transported across the cell monolayer from one surface to the opposite surface of the cell. This process may be important for the absorption of macromolecules (mainly

proteins) from the tubular lumen back to the blood (9), although transport in the opposite direction from basal to apical surfaces has also been demonstrated for epidermal growth factor (10). Several well established demonstrations of transcytosis in MDCK cells involve the use of transfected cells where specific receptors are expressed on either apical or basal cell surface. For example, in MDCK cells, transcytosis of membrane glycoprotein G from apical to basal surface has been demonstrated following implantation of the G protein from vesicular stomatitis virus into the apical plasma membrane (11). On the other hand, when the polymeric immunoglobulin receptor from rabbit is expressed in polarized MDCK cells, the receptor goes first to the basal surface and can be shown to transport dimeric IgA from the basal to the apical surfaces (4). Furthermore, the rates of endocytosis and transcytosis of fluid phase markers from both apical and basal surfaces have been examined (3).

Low density lipoprotein (LDL) receptors are widely distributed throughout the body. We carried out the study on this receptor to try to localize it on MDCK cells to determine its role in the control of cholesterol biosynthesis in relation to both the basal and apical cell surfaces and to examine the possibility that LDL might be transported across the epithelial monolayer in a manner similar to other proteins. Furthermore, we sought to determine whether such a transport system might be specific or not.

MATERIALS AND METHODS

Materials:

^{3}H-acetate (sodium salt; 130 mCi/mmole) and ^{3}H-inulin

(2.72 mCi/mgm) were purchased from New England Nuclear. Phosphate buffered saline (PBS, 10-fold concentrated), Earle's Minimum Essential Medium (MEM), bovine serum (FCS), penicillin-streptomycin (10,000 IU/ml and 10,000 μg/mg) and trypsin were obtained from GIBCO. Culture dishes and flasks were supplied by Becton Dickinson and Co.. Trichloroacetic acid (TCA), gelatin (300 Bloom, Type I) and heparin were purchased from Sigma Chemicals. $Na^{125}I$ was provided by Edmonton Radiopharmaceuticals in the Department of Pharmacy, University of Alberta. Polycarbonate membranes (25 mm diameter, 8 μm pore size) were obtained from Nucleopore.

Filter Unit:

Most experiments were done using filter units adapted in our laboratory. The unit was made by mounting a Nucleopore polycarbonate membrane filter (8 μm pores) onto a modified filtration unit from Millipore (25 mm). The filter unit was placed in a petri dish effectively producing two compartments, the lower compartment (basal) which has a volume of 10 ml and an upper compartment with a much smaller volume of 1 ml and comprising the apical surface. While the cells were normally grown attached to the filter in the upper compartment, they could also be grown in the lower compartment attached effectively to the filter in an upside down configuration, still retaining the basal surface attached to the filter but allowing for the apical surface to be exposed to the larger 10 ml volume (lower compartment) for greater sampling ease for the transport studies.

Cells:

The Madin-Darby canine kidney [MDCK (NBL-2)] cells were purchased from ATCC (line CCL34). Passages 63 to 68 were used in this study. Cells were first grown in monolayer in 75 cm^2 stock flasks containing 15 ml MEM supplemented with 10% FCS. Confluent monolayers from stock flasks were dissociated with 0.04% trypsin, 0.02% disodium ethylenediamine-tetraacetic acid (EDTA) buffered solution at 37°C for 10-20 min, and were seeded at a concentration of $2x10^6$ cells/cm^2 onto the filter units. Filter units were contained in 60 mm x 15 mm culture dishes for all of the studies. Prior to seeding the cells, the filter units with the filters mounted were coated and sterilized by boiling in 0.1% gelatin for 20 to 40 minutes. Non-sterile containers were sterilized by thoroughly washing with filtered water and placing in 75% ethanol for a short period of time. Cells were maintained in a temperature and humidity controlled incubator filled with 5% CO_2 and 95% air at 37°C for a minimum of 48 h prior to use unless otherwise specified. MEM containing 10% lipoprotein deficient serum (LPDS) which was prepared according to established procedures (1) was used to up-regulate LDL receptors 24 hours before the start of an experiment. Penicillin-streptomycin (the final concentrations: penicillin 100 IU/ml and streptomycin 100 μg/ml) was applied to all the culture media.

Lipoproteins:

Human LDL (density 1.019 - 1.063 g/ml) and human LPDS (density > 1.215 g/ml) were obtained from the plasma of healthy volunteers and prepared by differential ultracentrifugation (1). Human VLDL was prepared from the same plasma sample according to established procedures (12). The concentrations of LDL and VLDL are expressed in terms of protein content.

Cholesterol assays were also performed to assure the appropriate separation of the LDL and LPDS. The radio-iodination of LDL and VLDL with $Na^{125}I$ was done based on the procedure previously described (1). The preparation was passed through a Sephadex G-25 M column (Pharmacia PD-10) and dialyzed to remove free ^{125}I, yielding greater than 98% of TCA precipitable radioactivity. The solutions were filtered through a Millipore filter (Millex-GS, 0.22 μm) to get the final ^{125}I-LDL and ^{125}I-VLDL preparations.

Binding and Uptake from Different Surfaces:

Binding is defined as total association of ^{125}I-LDL with cells after incubation at 4°C for 2 hours (1.5 hours for ^{125}I-VLDL) followed by at least 3 washings with ice-cold PBS. Uptake measurements are made in a similar fashion but at 37°C for 2 hours. Uptake, therefore, consists of the components of both binding and internalization as well as degradation of the ^{125}I-LDL. For the binding or uptake on the apical surface, 1 ml of incubation medium containing ^{125}I-LDL (concentration as indicated) was added to the apical side. For that on the basal surface, the filter units were first turned upside down and then 1 ml of the incubation medium was added to the basal side of the cells. Binding was carried out on ice (4°C) and uptake in the incubator (37°C) for 2 hours. The nonspecific binding was determined in the presence of at least a 30-fold excess of unlabelled LDL or unlabelled VLDL in the case of ^{125}I-VLDL binding. The filters were washed by removing the filters from the holders and then dipping the filters using a pair of fine-tipped forceps successively in 3 beakers of ice-cold PBS (200-250 ml each).

The filters remained in each beaker for 10-15 sec with agitation. No radioactivity could be detected coming off of the filters in the third wash. More extensive washings did not result in changes in either specific or non-specific binding. The washing using different procedures will be described in the concerned legend. Protein assays determined that the filters contained 0.13-0.17 mg cell protein per cm^2 of filter when confluent high resistance monolayers were used.

LDL Receptor Regulation:

Cells were normally grown or maintained either in tissue culture flasks or on the filter units in the medium containing 10% FCS. Twenty-four hours prior to the experiments, the medium was changed to that supplemented with 10% LPDS or fresh FCS to up- or down-regulate the number of LDL receptors, as indicated. In some cases, LPDS (or FCS) was used to regulate the LDL receptor on a specific surface; this was done by adding LPDS- or FCS-containing medium to one of the chambers and the medium supplemented with FCS or LPDS to the other. The cells were washed with PBS at least twice before the addition of either fresh LPDS- or FCS-containing medium.

^{3}H-Acetate Incorporation into Cholesterol:

The incorporation study was carried out at 37°C for 3 hours. After regulation with LPDS and/or FCS on different surfaces for 24h, 100 μCi of ^{3}H-acetate was added to each unit (50 μCi to each side). Incorporation was terminated by removing the filters from the filter units, washing with ice-cold PBS 3 times and digesting the cells with 1 ml

of 1 N NaOH. 0.5 ml of each sample was taken for lipid extraction using a chloroform:methanol:water (2:1:1, v/v/v) system. Lipid phases were brought to dryness under N_2 and reconstituted with 100 μl of hexane. Thin-layer chromatography (TLC) (polygram Sil G, Maclerey, Nagel, Germany) was run to separate cholesterol from other non-polar and polar lipids. Samples were counted using 15 ml of ACS (Amersham). All values are expressed in terms of cpm ^{3}H-cholesterol produced per mg cell protein in the cell digest. Typically, triplicate samples were used for each condition and sample. Essentially identical results were obtained whether the ^{3}H-acetate was added to one or the other or both sides of the filters as long as the total amount was the same.

Transport of LDL across MDCK Monolayers:

Monolayers grown on filter units were used to look at the transport of LDL from the apical to the basal surfaces and from the basal to the apical surfaces. For examining apical to basal transport the filters were arranged with the filter units up and the apical surface exposed in the upper chamber. For examining transport from the basal to the apical sides the filter units with the cells plated normally were set up upside down with the basal surface through the filter exposed to the smaller volume in the upper chamber just before the transport study was to be carried out. By putting ^{125}I-LDL (35μg in 1 ml MEM) into the upper chamber and collecting 1 ml of aliquots from the lower chamber, we were able to easily measure the movement of the tracer ^{125}I-LDL across the monolayers both from the apical to the basal and from the basal to the apical surfaces. The experiments were carried out at 37°C with stirring

(using 3 mm x 12.5 mm Teflon micro-stirring bars in the lower chambers). Four-position multi-stirrers (Bellco Glass, Inc.) were used. The volume in the transport chambers was kept constant by adding the same amount of medium back to the containers when sampling at each time point. Similar data were achieved for the basal to apical transport when the filters were held right side up although a 10-fold greater amount of ^{125}I-LDL was required to be added to the lower chamber to effect the same concentration gradient (data not shown).

Detection and Exclusion of Leaky Monolayers:

For the transport study, continuity of monolayers was crucial. To screen the samples which are not leaky, ^{3}H-inulin was co-added with ^{125}I-LDL to the upper chambers and the amount of ^{3}H-inulin getting across the monolayers was monitored. Generally speaking, the passage of ^{3}H-inulin across confluent, non-leaking, high resistance monolayers is very low, a maximum of 0.07% for a 3 hour period. By comparing the movement of inulin with that of LDL we were able to exclude the samples with leaks which were sometimes generated by manipulations during the experiment. In these cases the monolayers would not distinguish between inulin or LDL movement. These amounted to no more than 10-20% of the filter preparations. The resistance of the cell monolayer was occasionally monitored according to established techniques (13) and yielded values in the order of 450 ohms.cm^2 after 24-48 h on the filters which is in agreement with published values (13) and measured using a Millicell-ERS system from Millipore.

Cell Solubilization:

For immunoblotting experiments, cell monolayers were washed, scraped, pelleted, and solubilized in the presence of 1.4% Triton X-100 by methods that have been previously described (14). Cell extracts were centrifuged at 300,000 x g for 40 min at 4°C and the supernatants were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis as described below.

Electrophoresis, Transfer to Nitrocellulose, and Immunoblotting:

Electrophoresis was conducted on 4.5-18% SDS-polyacrylamide gradient slab gels. Samples were prepared in the absence of reducing agents, gels were run and calibrated, and electrophoretic transfer to nitrocellulose was performed as described previously (14). Immunoblotting was performed in the buffer containing 25 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.05% Triton X-100, and 5% (w/v) non-fat dry milk as blocking agent (15). Visualization of bound rabbit IgG was with ^{125}I-labeled protein A. The concentration of the antibody and the specific radioactivity of ^{125}I-protein A used in the incubation mixtures are indicated in the legend to fig. II-5. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 films.

Other Methods:

The rabbit IgG fraction against bovine adrenal LDL receptor (anti-LDL-R) was raised as described in (16). ^{125}I-labeled protein A was obtained by the Iodogen method (17). IgG fractions were purified from sera on columns of protein A-Sepharose Cl-4B (15). The rabbit preimmune IgG used as a control to the anti-LDL-R was also purified on the column.

RESULTS

^{125}I-LDL Binding to Apical and Basal Surfaces after Receptor Regulation from Both Surfaces:

The LDL receptor in lymphocytes as well as in fibroblasts has been demonstrated to be down- or up-regulated (18) by the presence or absence of LDL. FCS containing LDL and LPDS containing no LDL were used to down- and up-regulate the receptor in MDCK cells. In the study shown in Fig. II-1, the same medium was used on both the basal or apical surfaces of the monolayers to examine regulation. After overnight incubations, binding was carried out at 4°C for 2 hours. There was no significant difference in the binding to the apical surface whether the cells were pre-incubated in LPDS or FCS (Fig. II-1). LPDS, however, produced a very substantial up-regulation of LDL binding to the basal surface. The data are expressed as ng of ^{125}I-LDL bound per mg cell protein and represents total binding. The fact that similar amounts of LDL bound to both basal and apical surfaces may be somewhat misleading since while the filters' surface areas were the same, the apical and basal surfaces may represent quite different surface areas and hence quite different binding densities.

^{125}I-LDL Binding to Basal Surface after Receptor Regulation from Different Surfaces:

MDCK Cells form a tight epithelial monolayer when allowed to grow to confluence. The confluent monolayer has a relatively high electrical resistance (446±8 ohms.cm^2 as determined for our monolayers) and a low permeability to large proteins such as LDL. Based on this property we

attempted to determine whether LDL receptors on the basal surface could be regulated by selectively adding LPDS or FCS to either or both bathing media and then examining ^{125}I-LDL binding to the basal surface. Fig. II-2 shows that the most effective way to down-regulate the LDL receptors on the basal surface is to apply FCS to both surfaces or to the basal surface alone keeping the apical surface bathed in LPDS. Similarly when LPDS is added to both surfaces or to the basal surface alone (keeping the apical surface exposed to FCS) the basal surface LDL receptor is up-regulated. Thus, it appears that the basal surface LDL receptor is regulated solely by the presence or absence of LDL in the medium bathing that surface.

Specific Binding of LDL to Apical and Basal Surfaces:

Fig. II-3 examines the total and nonspecific binding of ^{125}I-LDL to apical (panel A) and basal (panel B) surfaces as a function of ^{125}I-LDL in the apical or basal medium. Nonspecific binding was determined by adding a 30-fold excess of cold LDL. The figure shows a specific and saturable binding component on both surfaces although that to the basal surface is clearly larger Table II-1 demonstrates that the LDL binding to either surface likely represents the Apo B receptor as ^{125}I-VLDL binds similarly and can be displaced by either cold VLDL or cold LDL (19). This supports the notion that the LDL binding site on the apical surface is either the same or very similar to the basal surface receptor or traditional LDL receptor.

Inhibition of LDL Binding:

Fig. II-4 examines the ability of either cold LDL or antibody to the bovine LDL receptor (anti-LDL-R) to inhibit binding of ^{125}I-LDL to either basal or apical surfaces. Approximately 50% of the total ^{125}I-LDL binding was inhibited when either LDL or anti-LDL-R was added to the medium bathing either surface. This figure supports the notion that the LDL binding site on the apical surface represents an LDL receptor, at least one that cross reacts with the bovine LDL receptor (16). The basal receptor bears the same cross reactivity.

^{125}I-LDL Internalization from Different Surfaces:

Since LDL internalization is mediated primarily by the LDL receptor, it was of interest to determine the extent of internalization of ^{125}I-LDL from both apical and basal surfaces. ^{125}I-LDL uptake at 37°C was carried out from either surface with binding at 4°C from the same surface being used as control, since there would be no internalization expected at this temperature. The difference between uptake at 37°C and binding at 4°C roughly represents internalization. The net internalization of ^{125}I-LDL from basal surface amounts to 267.98 ng/mg cell protein in contrast to that from apical surface of 22.95 ng/mg protein (Table II-2). Given the differences in surface areas of the apical and basal surfaces this difference may vary if the data were expressed as a function of surface area rather than as a function of cell protein. There was no preferential degradation of ^{125}I-LDL (TCA soluble ^{125}I) when it was added from either the basal or apical surface and so all of the data are simply expressed as total TCA-precipitable ^{125}I-LDL associated with the cells following at least 3 washings.

Immunoblot Labelling of LDL Receptors:

In an attempt to identify the LDL receptor in the MDCK cells immunoblot experiments, utilizing the anti-LDL-R antibody, were carried out on solubilized cell monolayers from either tissue culture flasks (plastic) or membrane filters (gelatin-coated porous plycarbonate membrane, 8 μm pore size). When immunoblots were compared between FCS (Fig. II-5, lane 2) and LPDS (lane 3) treated cells very little regulation of LDL receptor could be seen for the cells grown on plastic dishes. This is likely due to the inaccessibility of the basal surface to the conditioning medium bathing the apical surface during the 24 hour period. When the cells were grown on filters with ready access of both surfaces to either FCS or LPDS clear regulation of the receptor is observed (lanes 4 and 5 for FCS and LPDS, respectively).

^{3}H-Acetate Incorporation into Cholesterol after Surface Selective LDL Receptor Regulation:

LDL taken up by the cell results in the release of exogenous cholesterol which in turn inhibits the endogenous synthesis of cholest and enhances its esterification when the cell's required complement of free cholesterol is met (20). Therefore, the availability of LDL to the cell through its receptor is inversely related to ^{3}H-acetate incorporation into cholesterol. In this study, we again selectively exposed either the apical or the basal surface or both surfaces to FCS or LPDS for 24 hours prior to determining ^{3}H-acetate incorporation into cholesterol. As in the case of LDL binding to the basal surface, Fig. II-6 shows that FCS added to both or just the basal surface results in almost

total suppression of cholesterol synthesis. The absence of LDL (incubation with LPDS) in the medium bathing either both surfaces or only the basal surface results in a sharp increase in ^{3}H-acetate incorporation into cholesterol regardless of whether the apical surface is exposed to FCS or LPDS. The control of cholesterol biosynthesis in the MDCK cells via the LDL receptor therefore appears to occur exclusively from the basal surface.

Table II-3 examines the incorporation of ^{3}H-acetate into cholesterol as a function of growth conditions. Confluent cells grown on plastic dishes have the lowest rate of synthesis and seem the least affected by the treatment with LPDS. This is likely due to the poor availability of the basal surface to up- or down-regulation by the medium. Non-confluent cells which would be expected to have a greater extent of exposure of the basal surface show a two-fold higher rate of cholesterol synthesis and a much greater stimulation in LPDS but still to a much smaller extent than seen for fibroblasts (20). The confluent cells grown on filters show by far the greatest stimulation by LPDS probably due to the free access of the basal surface for LDL receptor regulation by the FCS or LPDS from the lower chamber. This data is consistent with the notion that LDL receptor on the basal surface predominates in terms of the regulation of cholesterol biosynthesis.

Transport of ^{125}I-LDL across MDCK Monolayer:

From the experiments described above it appears that functional LDL receptors are found exclusively on the basal surface and are essentially absent from the apical surface except for a specific binding component that

has no apparent role in regulating cholesterol biosynthesis. Since we are interested in the transport of macromolecules (mainly proteins) across both epithelial and endothelial tissues, transport experiments were carried out to define the role of the asymmetrically distributed LDL receptors on LDL transport across the epithelial monolayers. The transport in both directions (i.e., from basal to apical surfaces and from apical to basal surfaces) was examined (Fig. II-7) both in the absence and presence of a 30-fold excess of cold LDL. The amount of ^{125}I-LDL transported is expressed in terms of ng/cm^2 where the area refers to the surface area of the filter support and not the cells. The figure suggests that whereas the transport from the apical side to the basal side appears to have a specific component, no such specific transport is seen for the movement from the basal to the apical surface.

Fig. II-8 demonstrates the temperature sensitivity of LDL transport from apical to basal surfaces under conditions when the cells are visually confluent but of low resistance versus cells seeded at the same density but allowed to sit for an additional 48 hours. Only when the cells have been confluent for 2 or more days is a sharp temperature dependence around 15-20°C seen, indicative of a transport system requiring active mechanisms such as receptor-mediated transcytosis in epithelial (thyroid follicle) cells (21) or endothelial tissue (22). When the cells are just recently confluent, ^{125}I-LDL transport is higher and shows a simple linear function of temperature, indicative of a simple diffusion or leak pathway, probably intercellular or paracellular. Both of these figures (II-5 and -6) strongly indicate the existence of a specific pathway responsible for the transport of LDL from the apical (lumen) to the basal surfaces (blood) side.

DISCUSSION

The data establish the point that functional LDL receptor is arranged in a highly polarized fashion across the epithelial (MDCK) cell in cultured monolayers. Two of the processes involved in cholesterol biosynthesis; ^{3}H-acetate incorporation into ^{3}H-cholesterol and LDL receptor regulation are influenced solely by the extent of the LDL composition in the medium on the basal surface, the side directed to the interstitial fluid and serum. Alterations in LDL levels in the apical medium appear to be without any effect on these processes. It is of interest to note that other receptors with similar functions such as the insulin receptor in rabbit kidney (23) and the transferrin receptor in MDCK cells (24) also have a polarized distribution being located predominantly on the basal surface. This stands to reason if one assumes that the epithelial cell receives its nutrients and essential elements like glucose, iron and cholesterol from the blood or basal side of the epithelium.

What then is the function of the LDL binding site on the apical surface and does it represent a true LDL receptor? Clearly this binding site or receptor is not involved in regulating cholesterol biosynthesis and its density does not appear to be regulated by the presence of LDL in the medium bathing either the basal or apical surface. The apical surface receptor is likely a typical LDL or Apo B receptor as indicated by its saturability, its specificity, the extent of interaction with VLDL and the ability of an anti-LDL receptor antibody to inhibit ^{125}I-LDL binding. Our data suggest that there is a specific mechanism for the transport of

LDL across the epithelial monolayer from the apical to the basal surfaces. It appears to be a specific transport mechanism that might be best explained by a process of "receptor-mediated transcytosis". Several points lead us to this conclusion: 1) The transport is unidirectional. While there is movement from the basal to the apical surface, it is non-specific and not temperature dependent (data not shown). 2) The transport from apical to basal surface shows a temperature sensitivity similar to the processes involving receptor-mediated internalization (21, 22). 3) The temperature sensitivity and specific transport require that the cells be confluent for a time similar to that required for the establishment of tight junctions and the development of a high resistance across the monolayer. The physiological function of such a transport mechanism is not clear since it is unlikely that any significant amount of LDL would be filtered at the glomerulus and hence be available to be transported from the lumen to the interstitial fluid and plasma. Nevertheless, tubular cells are known to transport a significant amount of protein. As much as 5 gm of albumin may be transported per day in a human kidney (25). LDL transport might represent a similar protein salvage system. It is of interest that receptor-mediated transcytosis in the apical to basal direction has also been postulated to occur for viral membrane glycoproteins implanted into the apical surfaces of MDCK cells (26, 11) while receptor-mediated transcytosis has been demonstrated in the basal to apical direction for IgA when MDCK cells were transfected to produce rabbit polymeric immunoglobulin receptors on the basal surface (Mostov & Deitcher, 1986). Similar basal to apical transport has been reported in MDCK cells for epidermal growth factor (10). Other authors have described transcytosis of macromolecules in

other types of epithelia including thyroid follicle cells (21) and newborn rat intestine (27).

It is also interesting to note that the movement of LDL from the apical to the basal surfaces appears to occur in the absence of a significant route involving its internalization and degradation. This is in contrast to the receptor on the basal side which binds LDL at 4°C and then is very active in internalizing the particle at 37°C presumably for the cell's own use and the regulation of cholesterol biosynthesis. We suggest therefore that LDL receptors (likely but not necessarily the same molecular species) exist on both sides of the polarized MDCK cell, but function differently. The receptor on the basal surface represents the traditional "Brown & Goldstein" receptor which can be regulated and which is responsible for the regulation of cholesterol biosynthesis. In contrast, the receptor on the apical surface, while specific, has none of these functions and appears solely responsible for the transport of LDL from the apical to the basal surface, perhaps representing a salvage pathway for filtered LDL.

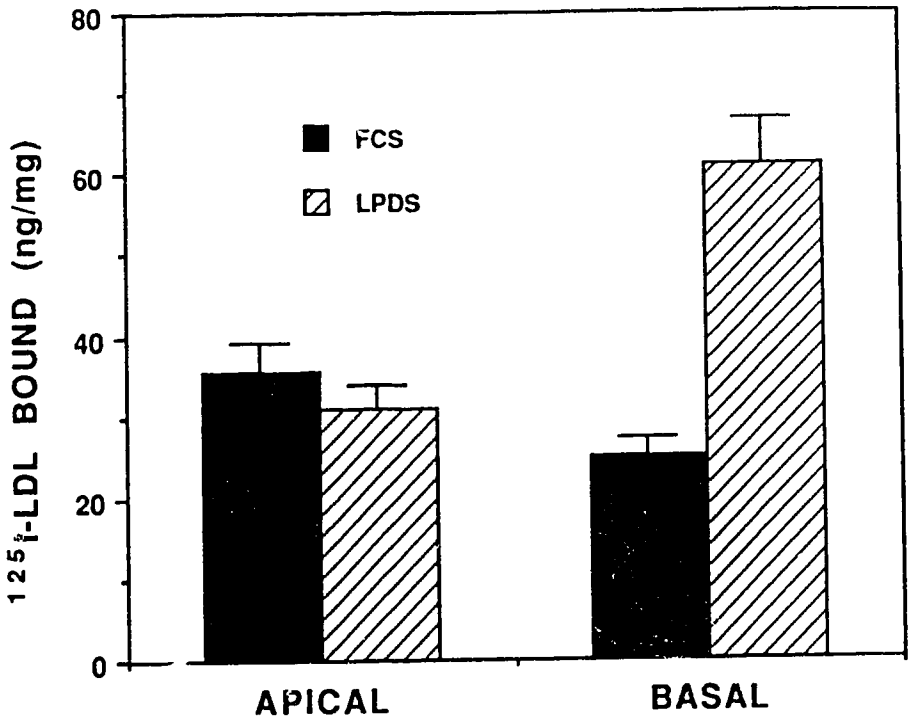


Figure II-1 The effects of FCS and LPDS on total LDL binding to basal and apical surfaces. MDCK cells were grown to confluence on the filter units in the MEM containing 10% FCS on both sides. The cells were then washed 3 times (both surfaces) with PBS and incubated for an additional 24 hours with the MEM supplemented with either 10% FCS or 10% LPDS on both the basal and apical surfaces. The cells were then cooled to 4°C, washed 3 times with ice-cold MEM. Binding of LDL was determined using 5 μg/ml of ^{125}I-LDL. The cells were solubilized using 1 N NaOH and the ^{125}I-LDL associated with the cells and total cell protein were determined. Each bar represents the mean and standard error from 4 separate samples (filter units). $p<0.01$ between the basal ^{125}I-LDL binding to the FCS-treated cells and that to the LPDS-treated cells by student t-test.

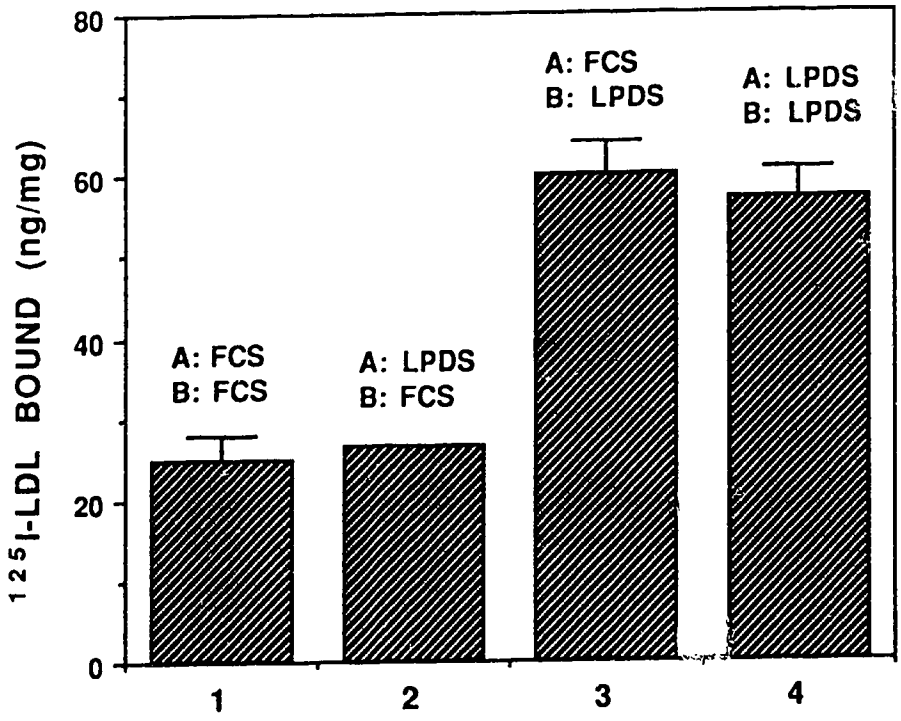
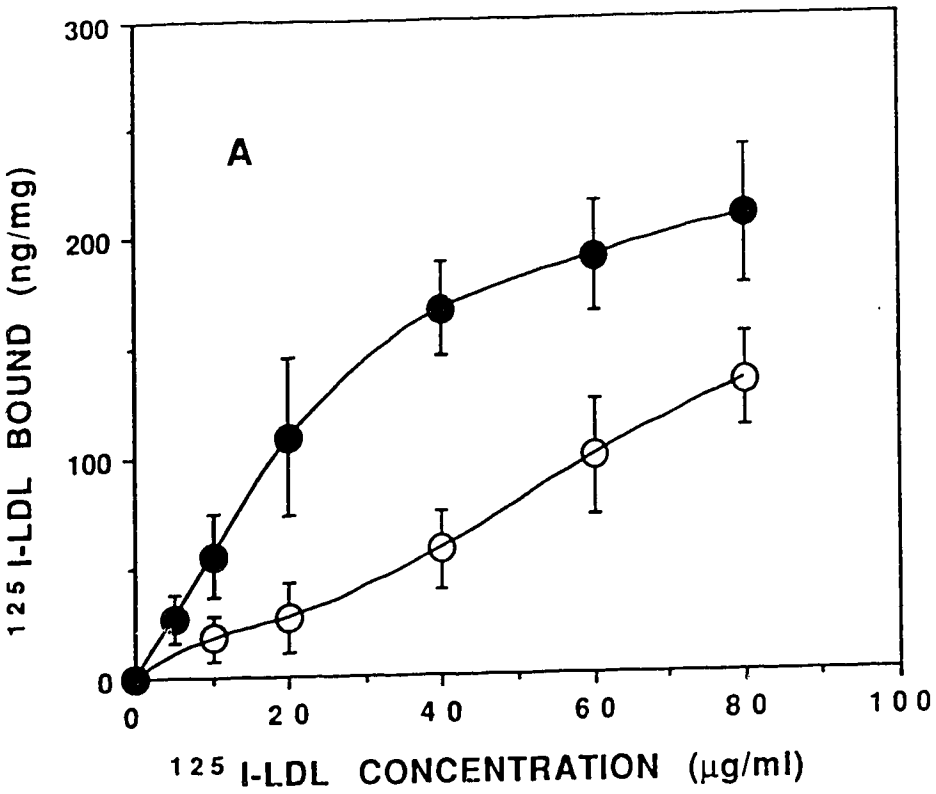


Figure II-2 The effects of FCS or LPDS in the basal and/or apical surface media on total LDL binding to the basal surface. Experiments were carried out essentially as described in Figure II-1 except that only LDL binding to the basal surface was measured as a function of FCS or LPDS in either or both of the basal and apical media. Each bar represents the mean and standard error from 4 separate samples (filter units).

Figure II-3 Total and non-specific binding of ^{125}I-LDL to either apical (panel A) or basal (panel B) surface of cultured monolayers of MDCK cells at 4°C. The cells were treated as described in Figure II-1 except that ^{125}I-LDL binding was performed with ^{125}I-LDL concentrations ranging from 5-80 μg/ml both in the absence (solid circles, for total binding) and presence (open circles, for nonspecific binding) of a 30-fold excess of cold LDL. The binding was carried out as described in the text and as in Figure II-1. Each point represents the mean and standard error from 3 separate filter units under identical conditions.


A
300
200
100
0
0
20
40
60
80
100
125 I-LDL BOUND (ng/mg)
125 I-LDL CONCENTRATION (μg/ml)

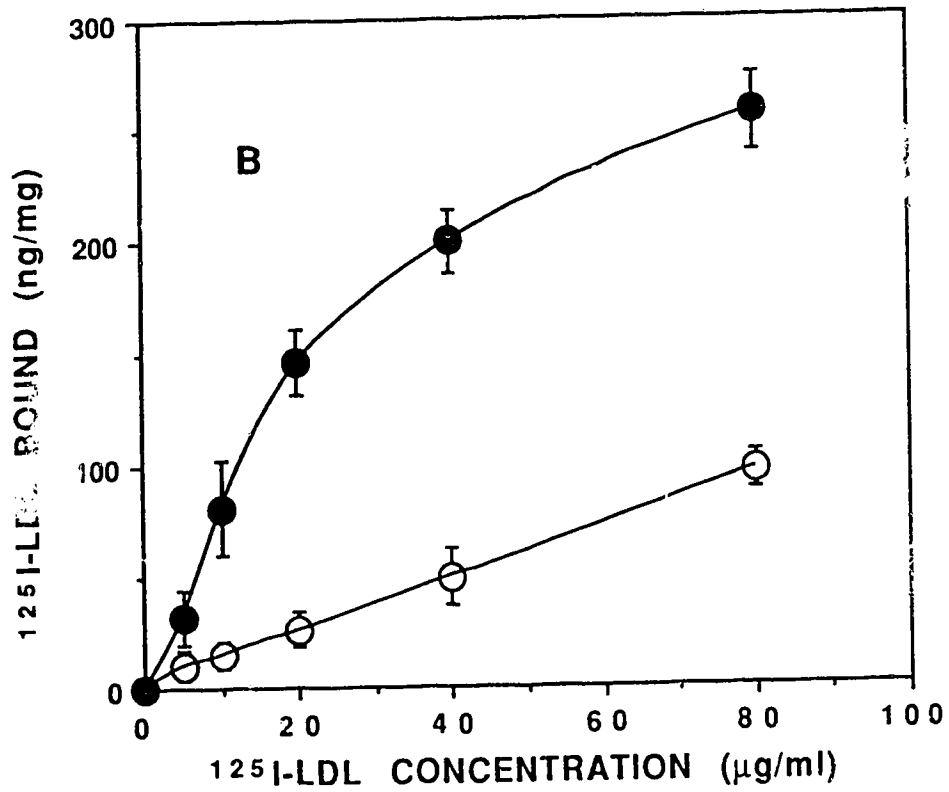
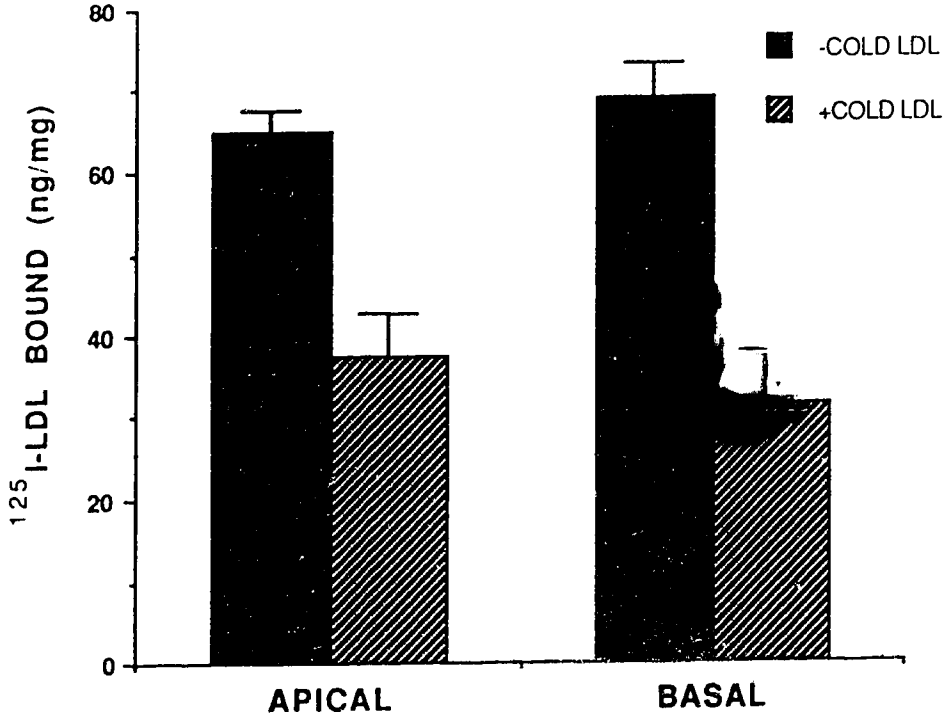

B
300
200
100
0
0
20
40
60
80
100
125 I-LDL BOUND (ng/mg)
125 I-LDL CONCENTRATION (μg/ml)

Figure II-4 The effects of cold LDL or anti-LDL-R antibody on the binding of ^{125}I-LDL in confluent MDCK monolayers. Cells were pre-incubated with 7% human LPDS for 24 hours prior to the binding experiments. The binding in the presence and absence of cold LDL (upper panel) was carried out at 4°C for 2 hours and that in the presence of anti-LDL-R and the control preimmune IgG was at the same temperature for one hour. 5 μg of ^{125}I-LDL in 1 ml MEM were added to either apical or basal side of each sample. A 30-fold excess of cold LDL, and 1.5 mg of anti-LDL-R and preimmune IgG were added to the indicated samples. The washing was done differently from the other experiments. The filters were taken off the holders and put into dishes. 10 ml PBS were added to each dish containing the filter and remained with agitation for 30 minutes. The same washing process was repeated 3 times. The radioactivity associated with the cells was determined. n = 3 and $p<0.01$ between the binding to the FCS-treated cess and that to the LPDS-treated cells for all the four pairs of data by student t-test.


80
60
40
20
0
^{125}I-LDL BOUND (ng/mg)
-COLD LDL
+COLD LDL
APICAL
BASAL

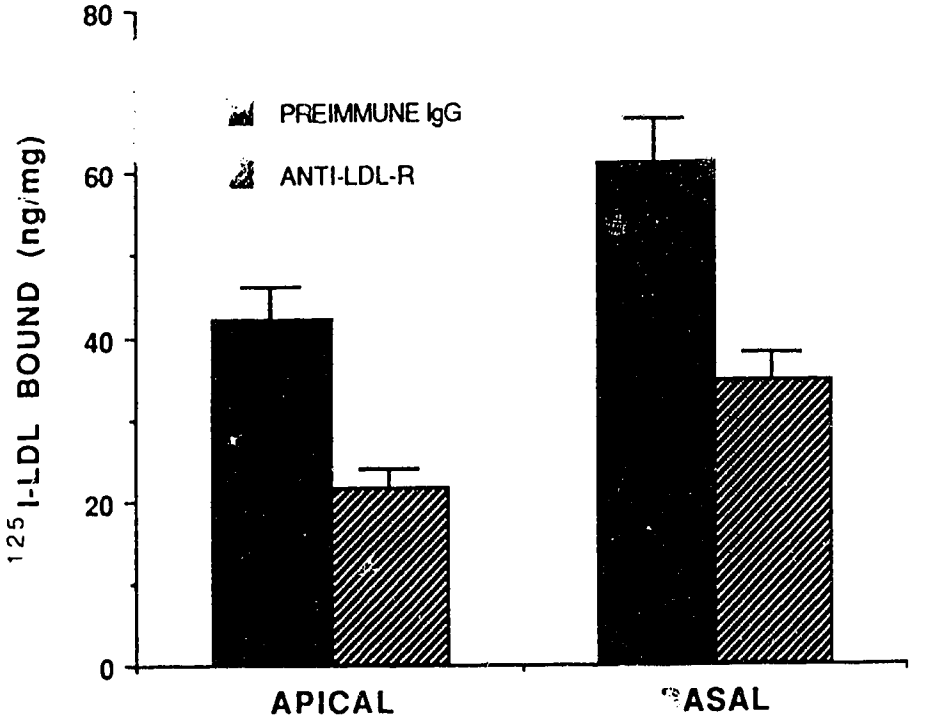
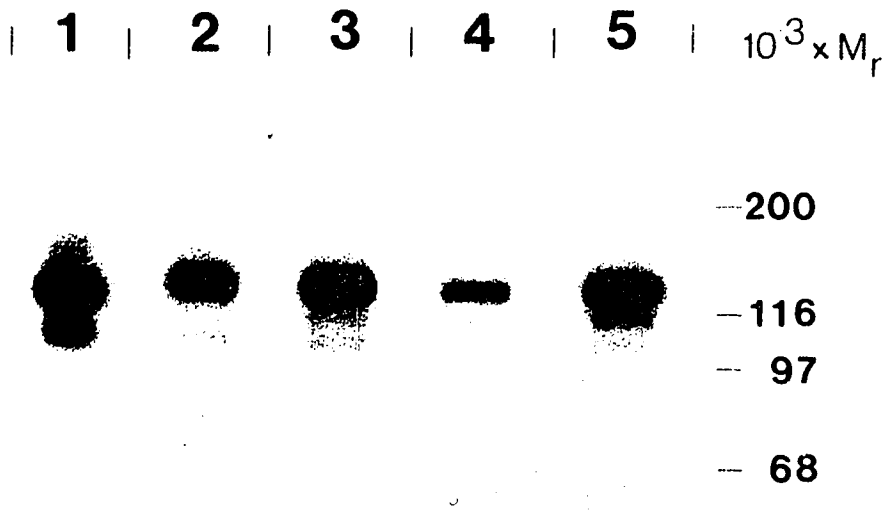

80
60
40
20
0
^{125}I-LDL BOUND (ng/mg)
PREIMMUNE IgG
ANTI-LDL-R
APICAL
BASAL

Figure II-5 Visualization of LDL receptor by ·noblotting. MDCK cells were cultured as described in Materials and Methods. For the last 24 hours before harvest, cells both grown on filters and petri dishes received medium containing 10% LPDS alone (LPDS) or 10% FCS in the presence of 12 μg/ml of cholesterol and 2 μg/ml of 25-OH-cholesterol (FCS). Lane 2 represents the cells prepared on dishes treated with FCS, lane 3 the cells on dishes treated with LPDS, lane 4 the cells on filters treated with FCS and lane 5 the cells on filter treated with LPDS. Cell pellets were solubilized as described in Materials and Methods and the resulting supernatants were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated in the presence of 10 μg/ml of anti-LDL-R IgG, followed by ^{125}I-labelled protein A (1.6 μg/ml; 206 cpm/ng) as described in Materials and Methods. Lane 1 contained 100 μg of detergent-solubilized membrane proteins from bovine adrenal cortex, prepared as described in Materials and Methods. Autoradiograhy was for 28 hours at -70°C.


1
2
3
4
5
$10^3 \times M_r$
200
116
97
68

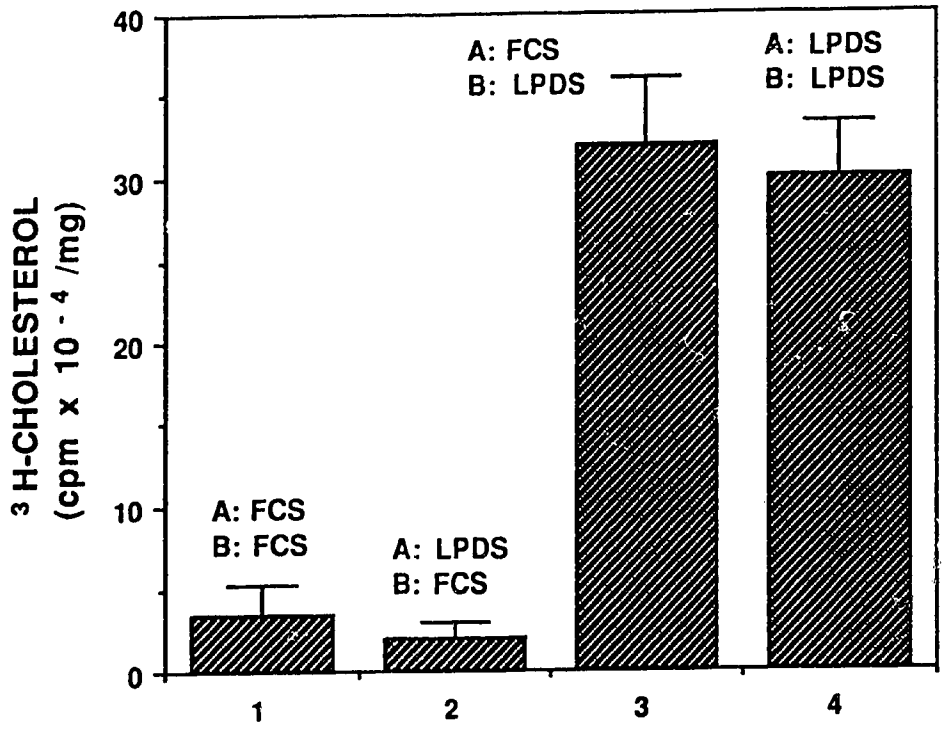
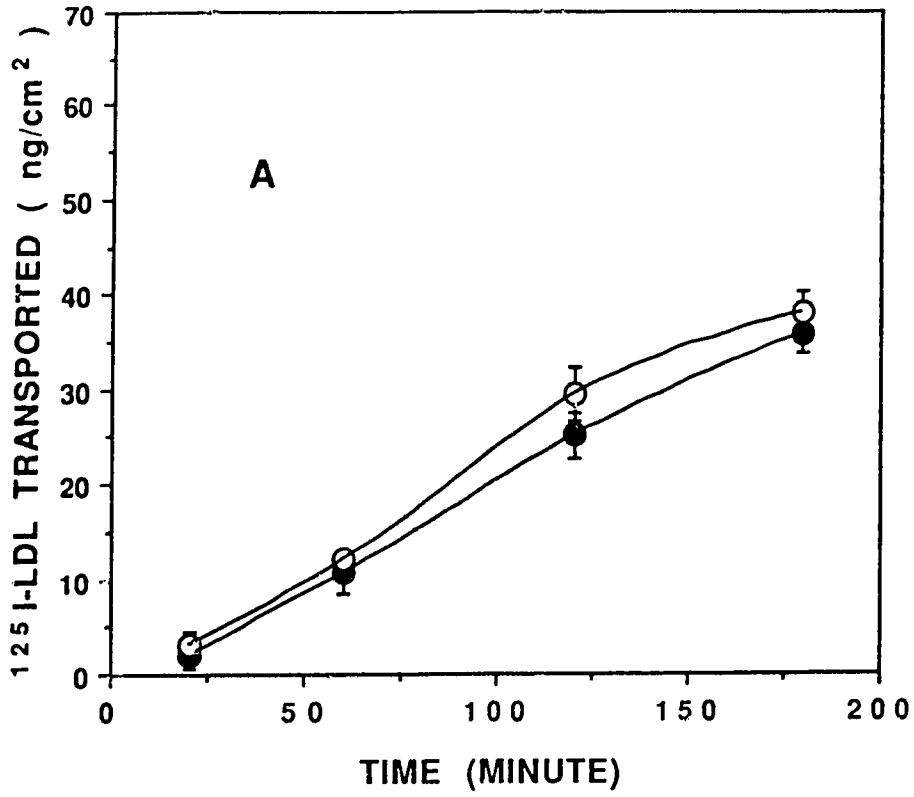
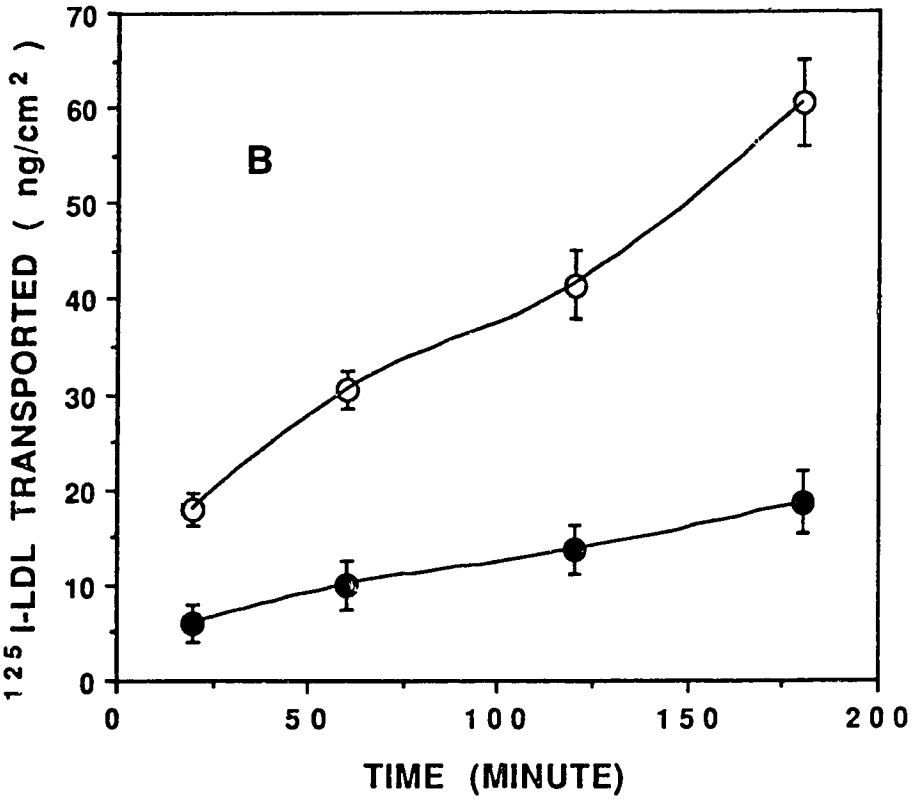


Figure II-6 ^{3}H-acetate incorporation into cholescerol as a function of FCS or LPDS present in either or both of the basal and apical surface media. The cells were prepared as in Figure II-1. Following the 24 hour incubation of the cells with FCS or LPDS, ^{3}H-acetate was added to both the upper and lower chambers (50 μCi to each) and the cells were kept at 37°C for an additional 3 hours. The cells were then washed at least 3 times with ice-cold PBS and solubilized using 1 N NaOH. Lipid extraction was followed by TLC in order to isolate the cholesterol and determine radioactivity by liquid scintillation counting. Each bar represents the mean and standard error values for 3 samples.

Figure II-7 Transport of ^{125}I-LDL (35 μg/ml) across confluent monolayers of MDCK cells from either the apical to the basal surfaces (panel B) or from the basal to the apical surfaces (panel A). The radiolabelled LDL was added to one surface at time 0 and then samples were taken from the medium bathing the opposite surface at regular intervals. The open circles in both panels represent ^{125}I-LDL transported in the absence of any cold LDL whereas those with the closed circles represent the amount of ^{125}I-LDL transported in the presence of a 30-fold excess of cold LDL. The data is expressed as ng of ^{125}I-LDL transported per unit area represented as the area of the filter.


A
^{125}I-LDL TRANSPORTED (ng/cm^2)
0
10
20
30
40
50
60
70
0
50
100
150
200
TIME (MINUTE)


B
^{125}I-LDL TRANSPORTED (ng/cm^2)
0
10
20
30
40
50
60
70
0
50
100
150
200
TIME (MINUTE)

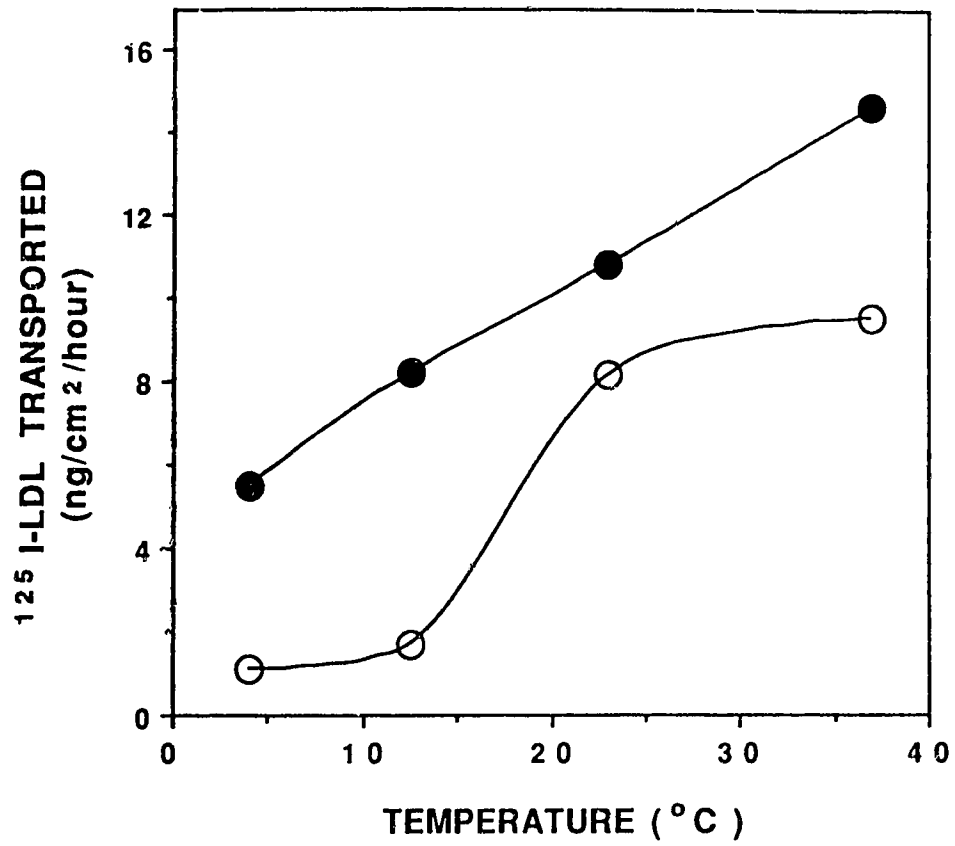


Figure II-8 The effect of temperature on the transport of ^{125}I-LDL (10 μg/ml) from apical to basal surfaces. The cells were seeded on the membrane surface in a confluent fashion at a density of $4x10^6$ cells/cm^2. In one instance transport studies were carried out when the cells had been on the filters for only 18 hours (solid circles) whereas in the second instance, the cells were allowed to remain on the filter for 3 days prior to the transport study being carried out (open circles). The cells were washed from both sides, incubated with MEM and temperature adjusted for at least 20 minutes at one of the designated temperatures prior to the transport of ^{125}I-LDL being carried out at that temperature in a manner analogous to that described for Figure II-7. The data represent the means for 3 filters or samples determined at each temperature. Standard errors ranged from 2-15% of the mean.

Table II-1 ^{125}I-VLDL Binding to Apical and Basal Surfaces

	Apical Surface	Basal Surface
	ng ^{125}I-VLDL/mg protein	
Exp. 1		
^{125}I-VLDL	105.1 ± 5.9*	88.3 ± 4.8
^{125}I-VLDL + Cold VLDL	14.5 ± 1.2	14.1 ± 1.2
Expt. 2		
^{125}I-VLDL	91.9 ± 1.6	
^{125}I-VLDL + Cold LDL	34.5 ± 0.6	

Cells were grown on Nucleopore membranes and prepared as described in the text. Following a 24 hour incubation in LPDS the binding of ^{125}I-VLDL (5 μg/ml) to either apical or basal surfaces was carried out for 1.5 hours at 4°C. Cold VLDL (Expt. 1) or LDL (Expt. 2) was added at a concentration of 200 μg/ml at the same time as the ^{125}I-VLDL.

*Data is expressed as ng/mg total cell protein ± S.E. from triplicate samples for each condition. Each Nucleopore membrane had between 0.13 and 0.17 mg cell protein per cm^2.

Table II-2 ^{125}I-LDL Internalization From Apical and Basal Surfaces

	From Apical Surface	From Basal Surface
	ng ^{125}I-LDL/mg protein	
Uptake at 37°C	54.38 ± 5.18*	322.41 ± 67.40
Binding at 4°C	31.43 ± 3.10	54.43 ± 5.93
Net Internalization	22.95	267.98

Confluent MDCK monolayers were prepared on Nucleopore membrane filters in MEM containing 10% FCS as described. The cells were washed (both sides of filter) and reincubated for an additional 24 hours in MEM plus 10% LPDS. Binding and uptake studies were then carried out using 10 μg/ml of ^{125}I-LDL to either the apical or basal surfaces; binding for 2 hours at 4°C and uptake for 2 hours at 37°C as described in the Materials and Methods section to include both internalization and degradation.

*Data is expressed as ng/mg cell protein ± S.E. Each filter contained between 0.63 and 0.83 mg of cell protein or 0.13 to 0.17 mg cell protein per cm^2.

Table II-3 ^{3}H-Cholesterol Biosynthesis

MDCK Monolayer Preparation	Media		
	cpm(^{3}H-cholesterol)/mg protein		
	FCS	LPDS	LPDS/FCS
Confluent on dishes	5343 ± 273*	8074 ± 301	1.51
50% confluent on dishes	11051 ± 706	52728 ± 2855	4.77
Confluent on filters	7900 ± 4251	254699 ± 1369	32.24

Cells were grown on culture dishes or Nucleopore filters at various densities in MEM containing 10% FCS for aminimum of 3 days. The cells were then washed and media containing either 10% FCS or 10% LPDS added for an additional 24 hours. They were washed again and ^{3}H-acetate was added in MEM for a period of 3 hours before the cells were solubilized with 1 N NaOH, the lipids extracted and thin-layer chromatography performed to determine the ^{3}H-cholesterol synthesized.

*Data is expressed as cpm(^{3}H-cholesterol)/mg cell protein ± S.E. synthesized from ^{3}H-acetate.

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CHAPTER III

CHARACTERIZATION OF THE ZO-1 PROTEIN IN ENDOTHELIAL AND OTHER CELL LINES

KEY WORDS:

ZO-1 protein

endothelial monolayer

confluency

tight junction

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INTRODUCTION

The *zonula occludens* (ZO) or tight junction is apparently responsible for establishing high resistance junctions and sealing the intercellular spaces between cells in continuous monolayer both *in vivo* and in tissue culture. It has been shown conclusively to be present in most epithelia and certain types of endothelia such as brain vascular endothelium (Rutten *et al.*, 1987) which can develop a high transcellular electrical resistance in confluent monolayer form. It has been suggested that tight junctions may also be present in low resistance generating endothelia such as the aortic endothelium (Albelda *et al.*, 1988).

There are several hypotheses regarding the composition of the tight junction. One suggests that the junction is formed with the participation of specific proteins (Griepp *et al.*, 1983; Staehelin *et al.*, 1969). This hypothesis had not been biochemically substantiated until the demonstration of the ZO-1 protein. Stevenson and Goodenough isolated a detergent-insoluble tight junction-enriched fraction from mouse liver (Stevenson and Goodenough, 1984). This fraction was then used to generate a number of monoclonal antibodies which specifically react with a high molecular weight (225 Kd and 210 Kd depending on tissue and cell type) tight junction-associated peptide named ZO-1 (Stevenson *et al.*, 1986; Anderson *et al.*, 1988). The antibodies cross-react with several tissues (and between species) containing tight junctions and are localized at the junctional complex region of a number of epithelia, including colon, kidney ar testis, and to arterial endothelium by

immunofluorescence staining of sections of whole tissue (Stevenson *et al.*, 1986). The antibodies also stained the junctional complex region in a confluent monolayer culture of MDCK epithelial cells (Stevenson *et al.*, 1986; Stevenson *et al.*, 1988b). The ZO-1 protein was optimally soluble in 6 M urea or high pH , partially soluble in 0.3 M KCl and essentially insoluble by nonionic detergents, suggesting that the protein is a peripherally-associated membrane protein. The protein is an asymmetric monomer in purified form and is phosphorylated at serine residues (Anderson *et al.*, 1988). For a more detailed review, see Stevenson *et al.* (1988a).

It has been demonstrated that the distribution and content of the protein bear no relationship with the extent of transcellular electrical resistance in two different lines of MDCK cells (Stevenson *et al.*, 1988b). On the other hand, Anderson and co-workers (1989) have shown that when Caco-2 cells in suspension culture were replated to form confluent monolayers there was a dramatic increase in the expression of the protein ZO-1 with growing time. In this study, we attempted to correlate the content of ZO-1 protein with monolayer confluency of bovine aortic endothelial cell cultures and to examine the presence of the protein in some other types of cell cultures available in the laboratory.

MATERIALS AND METHODS

Monoclonal Antibody

The monoclonal antibody produced against a canalicular-enriched membrane fraction from mouse liver (anti-ZO-1) was kindly provided by Dr. Stevenson from Department of Anatomy and Cell Biology of the University of

Alberta. Detailed methods for its production have been described previously (Stevenson *et al.*, 1986). Several versions of anti-ZO-1 have been reported, including R26.4C, R40.40D3 and R40.76. We were provided with R40.76. The antibody was contained in culture medium RPMI 1640 which was used directly without further purification.

Cell Cultures

Most cell cultures were purchased from American Type Culture Collection, Bethesda, MD, including bovine aortic endothelium, rat intestinal epithelial cell (IEC-6) and MDCK cells. A mouse myeloma cell line was a gift from Department of Immunology at the University of Alberta. All the cells were grown in minimum essential medium (MEM) with 10% FCS (both from GIBCO) and penicillin-streptomycin (100 IU/ml and 100 μg/ml, Sigma) added except for myeloma cells which were grown in RPMI 1640 (GIBCO) with 10% FCS and without penicillin-streptomycin. The cultures were contained in either 75 cm^2 tissue culture flasks or 500 cm^2 culture trays. In the studies examining the ZO-1 protein as a function of the extent of confluency of endothelial cells, the cells were either seeded at different densities and harvested at the same time or seeded at the same density and harvested at different times to achieve different degrees of confluency.

Trypsinization (0.04% trypsin in calcium-free Puck's saline with EDTA) was used to collect endothelial, IEC-6 and MDCK cells for reseeding. Cells were harvested with a rubber policeman for the experiments.

Immunofluorescence Labelling of Monolayers

Endothelial, IEC-6 or MDCK cells were grown to confluence in Lab-Tek tissue culture chamber/slides (Nunc Inc.) coated with polylysine (100 μg/ml, Sigma). The chamber/slides were thoroughly washed with FCS-free MEM before cells were seeded. Confluent cells were fixed for 20 min with fresh 4% paraformaldehyde, made up as follows. 50 ml of a 2-fold concentrated phosphate buffered solution (192 mM NaOH, 244 mM NaH_2PO_4, pH 7.0-7.2) was added to 40 ml of distilled water and heated to 60°C. 4 g of paraformaldehyde (Sigma) was added with stirring until it was dissolved and then 5 ml of 100 mM $MgCl_2$ was added. The volume was brought to 100 ml, then filtered. Cells were permeabilized with fresh 0.1% Triton X-100 (Fisher) in Dulbecco's phosphate buffered saline (PBS) for 5 to 10 min. They were washed with the PBS 5 times both before and after permeabilization. The above treated monolayers were first incubated with medium A (MEM plus 10 % FCS) for 20 min. The incubations with antibodies and fluorescent label occured in the following order: the first incubation using anti-ZO-1 containing RPMI 1640 medium plus 10% FCS was carried out for 2 hours. The second incubation used 7.5 μg/ml of biotinylated second antibody rabbit anti-rat IgG (Vector) in medium A and was allowed to proceed for an additional 2 hours. The third incubation was carried out in dark with medium A containing 4 μl/ml of Texas red-conjugated streptavidin (Amersham) for 1 hour. The cells were washed with MEM plus 5% FCS 5 times between each of the above incubations. Final wash after streptavidin was with PBS (5 times). All of the above treatments were carried out at room temperature. The plastic chambers were taken off the slides before the latter were mounted. The slides can be stored at 4°C in the dark for extended periods of time.

SDS-PAGE

The ZO-1 protein was separated by running a 4.5 % to 18% gradient SDS-PAGE on a Protean II^{TM} slab cell electrophoresis apparatus (Bio-Rad), unless otherwise specified. Cells were harvested with a rubber policeman (for endothelial, IEC-6 and MDCK cells) in PBS or collecting the culture media directly (for myeloma cells). Cell suspensions were centrifuged at 2,000 x g for 15 minutes to obtain cell pellets. Urea extraction of the ZO-1 protein was done at 4°C in the buffer containing 10 mM Tris HCl, 1 mM EDTA, 6 M urea and the following protease inhibitors: phenylmethyl sulphonyl fluoride (1 mM), aprotinin (5 μg/ml), leupeptin (4 μM) and trypsin-chymotrypsin inhibitor (20 μg/ml). All the inhibitors were purchased from Sigma. The pellets in the extraction buffer were first passed through a 22 G syringe needle 10 times before sonication with a probe sonicator for 1 minute. The syringe passing was repeated. Essentially all the cells were broken by these treatments as examined by phase contrast microscopy. The extracts were centrifuged at 90,000 x g for 1 hour at 4°C on a Beckman 55 Ti swinging bucket rotor. The supernatants were transferred to other tubes and mixed with equal volumes of a 2-fold concentrated buffer made of 20% glycerol, 4.6% SDS and 125 mM Tris base with 100 mM dithiothreitol before loading onto the SDS gel. Protein was measured by Lowry assay (Lowry *et al.*, 1951). SDS-PAGE was also run with whole cells by following Stevenson *et al.* (1986).

Electrical Protein Transfer and Immunoblot

Proteins were transferred from the gels onto nitrocellulose membranes (Trans-blot Transfer Medium, 0.45 micron, Bio-Rad) (Howe and Hershey,

1981) by using a Bio-Rad Trans-blot apparatus in the buffer containing 20% methanol, 150 mM glycine and 20 mM Tris base. The transfer was done at 60 volts for overnight at room temperature. Immunoblotting of the membranes was done at room temperature. After the incubation with the anti-ZO-1 containing medium (undiluted), the nitrocellulose membranes were treated with a horseradish peroxidase-conjugated second antibody goat anti-rat IgG (Sigma, used as 1:300 dilution) in Buffer A (50 mM Tris HCl, 2 mM $CaCl_2$, 100 mM NaCl, pH 8.0). All the above incubation buffers contained 5% fat-free milk powder (Carnation, Inc.). Color development was with 5-chloro-1-naphthol (Sigma, 0.5 mg/ml in milk-free Buffer A containing 20% methanol and 0.05% H_2O_2).

RESULTS

Immunofluorescence Labelling of ZO-1 in Endothelial, IEC-6 and MDCK Cells

Immunofluorescence labelling of the protein ZO-1 in endothelial cells was conducted on two types of monolayers: confluent and nonconfluent. As shown in Fig. III-1 the protein is extensively expressed in cell peripheries in the confluent monolayer (A). The label outlined each cell clearly. In contrast, the cell peripheries in the nonconfluent monolayer (B) generally lacked the label except in the regions where cell-cell contacts occurred. In both types of cell preparations, there was weak labelling of the protein in the cytoplasm of the cells, which enabled us to visualize each of them. From comparing the cytoplasmic labelling of the two types of cells, it seems that confluent cells had more ZO-1 not only in peripheries but also in cytoplasm over nonconfluent ones. The pattern of labelling in endothelial cells is somewhat different from that in

epithelial cells as shown in Fig. III-2 and shown by others (Stevenson *et al.*, 1988b). The junctional regions were delineated by the ZO-1 label as an intercalated line in endothelial cells whereas the label in epithelial cells is a straight line. The significance of this difference is not known. The results for IEC-6 cells is shown in Fig. III-2, A. Cells were outlined by the ZO-1 label. Immunofluorescence studies on MDCK cells have been reported (Stevenson *et al.*, 1986; Stevenson *et al.*, 1988a and 1988b) and were used in our experiments as a positive control (Fig. III-2, B). Of the three types of cultures, MDCK gave the strongest labelling, IEC-6 the weakest with endothelial cells in between the two. The order is in good agreement with the immunoblotting results in Fig. III-5.

Monolayer Confluency vs ZO-1 Expression

Studies on the relationship between ZO-1 expression and monolayer confluency were carried out on endothelial cells, in which our laboratory has an interest in terms of the transcellular transport of large macromolecules. Cell monolayers with different confluencies were achieved either by seeding at the same density and growing for different lengths of time (Fig. III-3) or by seeding at different densities and growing for the same period of time (Fig. III-4). SDS-PAGE and immunoblotting of protein ZO-1 were carried out by using urea extracts of the cells and whole cells, the former allowing larger sample load on SDS-PAGE as well as giving sharper bands on immunoblot and the latter better reflecting the protein content of the cells. As seen in Fig. III-3, A, the expression of ZO-1 protein by immunoblotting is correlated with monolayer confluency which is

shown by phase contrast photomicrographs of the cells in Part C. Coomassie Blue stained gel is also given to show the profile of total protein loaded onto the SDS gel (Fig III-3, B). Fig. III-4 shows the relationship between the ZO-1 expression and the degrees of monolayer confluency, which were achieved by seeding the cells at different densities and growing for the same length of time. A similar pattern of confluence-related expression of the ZO-1 protein is observed in both the immunoblot transferred from the SDS-PAGE of urea extracts (Panel A) and the immunoblot transferred from that of the whole cells (B). The amount of samples loaded onto SDS gel was based on protein for the former and on cell number for the latter. There is no noticeable difference between the two procedures in terms of demonstrating ZO-1 expression-monolayer confluency relationship. It implies that the possibility of ZO-1 having different urea extractability under different monolayer confluencies is essentially ruled out.

ZO-1 IN OTHER TYPES OF CELLS

We examined the presence of the ZO-1 protein in a number of cell cultures available to us. They were two epithelia: MDCK from canine kidney tubule and IEG-6 from rat intestine; one endothelial line from bovine aorta; and one mouse myeloma cell line. ZO-1 was found to be present in endothelial cells and IEC-6 cells by immunoblotting (Fig. III-5, lanes 1 and 2 in A and B), as shown by immunofluorescenec labelling of the cells (Fig. III-1 and Fig. III-2, A). MDCK cells also gave a positive immunoblot (Fig. III-5, lanes 3 in A and B), which has been reported previously (Stevenson *et al.* 1986; Stevenson *et al.*, 1988a and 1988b;

Anderson *et al.*, 1988). Fig. III-5, B is the result of a non-gradient SDS-PAGE instead of a gradient one as used in other immunoblotting experiments to give a more accurate estimation of molecular weight. The molecular weight of the protein in the three types of cells which gave positive immunoblot results appears similar at 210 Kd on both gradient (Fig. III-5, A) and non-gradient gels. On the other hand, mouse myeloma cells (lane 4 in Fig. III-5, A) gave a negative result. Myeloma cells were grown in suspension and did not develop intercellular tight junction. The absence of the protein in this cell type would provide evidence for the association of the ZO-1 with tight junction.

DISCUSSION

The main purpose of these studies was to characterize the ZO-1 protein in vascular endothelial cells with emphasis on the relationship between the protein content and monolayer confluency. Based on previous studies (Stevenson and Goodenough, 1984; Stevenson *et al.*, 1986; Anderson *et al.*, 1988), it appears that this protein is universal among epithelial cells. This has also been demonstrated using immunofluorescence techniques in arterial endothelium of whole tissue sections (Stevenson *et al.*, 1986).

Even though epithelial and endothelial tissues have different embryological origins, they share some common properties: formation of physiological monolayers, function as a division between two different environments and development of transcellular electrical resistance in cultured monolayer (Rutten *et al.*, 1987; Albelda *et al.*, 1988 for endothelium). The presence of tight junctions between epithelial cells has

been well established. There is also a general agreement that brain vascular endothelium, which represents a specialized high transcellular resistance barrier, also develops an intercellular tight junction (Dorovini-Zis *et al.*, 1987). However, the presence of tight junctions in other types of endothelia including that from the bovine aorta remains controversial. Although the exact role of the ZO-1 protein has not been established, it is clear that the protein is associated with tight junctions. The presence of the ZO-1 in cultured cells and in cells of whole tissue sections (Stevenson *et al.*, 1986) provides evidence for the existence of tight junctions in endothelium, as hypothesized (Poznansky and Juliano, 1984). Failure to demonstrate the presence of the protein in myeloma cells (Fig. III-5) seems to make this protein unique to epithelium and endothelium. It is reasonable to believe that only tight junction-containing cell types should express the protein whereas cells like myeloma which do not have tight junction should not. However, we can not make a firm conclusion until a variety of non-tight junction-forming cells have been examined to assure the negative results.

We demonstrated a strong correlation between the amount of the ZO-1 protein and monolayer confluency (Fig. III-1, III-3, III-4) in the endothelial cell preparation. A similar result was shown in human intestinal epithelial line Caco-2 cells (Anderson *et al.*, 1989). Cells were first grown in suspension for 48 hours in Ca^{++}/Mg^{++}-free spinner medium during which time they lost cell-cell contacts. They were then replated at confluent density in Ca^{++}/Mg^{++}-containing medium so that fully confluent monolayers formed shortly. When the expression of the protein ZO-1 was examined against monolayer growing time, there was a

steady increase up to two weeks. We found that expression of ZO-1 protein in cell periphery in endothelial cells only occurred in the region where cells had contact with each other (Fig. III-1). It seems that the same thing happens in MDCK cells (Siliciano and Goodenough, 1988). The difference in monolayer confluency in our experiments and monolayer growing time in theirs may have the same consequence: altered cell-cell contact. All these results support the notion that cell contact is needed for the expression of the protein.

The molecular weight of ZO-1 in mouse liver and kidney was shown to be 225 Kd and in MDCK cells to be 210 Kd (Anderson *et al.*, 1988). Our study agrees with the 210 Kd reported in MDCK cells. We also found that ZO-1 in endothelium and IEC-6 seems to have the same 210 Kd value.

The reason for using urea extracts to run SDS-PAGE and immunoblots is that larger amounts of ZO-1 protein could be loaded onto SDS gel, which is especially important for examining the presence of the protein in the myeloma cells. Also it resulted in sharper bands. The cells under different degrees of confluency seemed to have no difference in urea extractability of the protein ZO-1, since on the basis of per number of cells the protein showed similar pattern of confluence-related expression (Fig. III-4).

In summary, a few points might be concluded from this study: bovine aortic endothelial cells, representative of low resistance endothelia, do have ZO-1, indicative of the existence of tight junctions. The content of the ZO-1 protein in endothelial cells is positively related to monolayer confluency. It seems that the ZO-1 protein is unique to epithelium and the equivalent endothelium, while being absent from the cells like myeloma which grow in suspension culture.

Fig. III-1 Indirect immunofluorescence labelling of ZO-1 protein in endothelial monolayers. Confluent (A) and nonconfluent (B) monolayers were prepared by plating the cells at different densities and growing for 1 day before use. Labelling was carried out as described in Materials and Methods. Exactly the same experimental and photographic conditions were used for the two monolayer preparations. Cell peripheries were intensively labelled for the protein in the confluent monolayer whereas the peripheries in nonconfluent monolayer were lacking of labelling except those in the regions where cell-cell contacts occurred. In the latter case, most of the cell peripheries are difficult to identify on the fluorescence photomicrographs (B) even though the cells as a whole can be seen clearly. Cytoplasmic labelling occurred in both the confluent and nonconfluent cells.

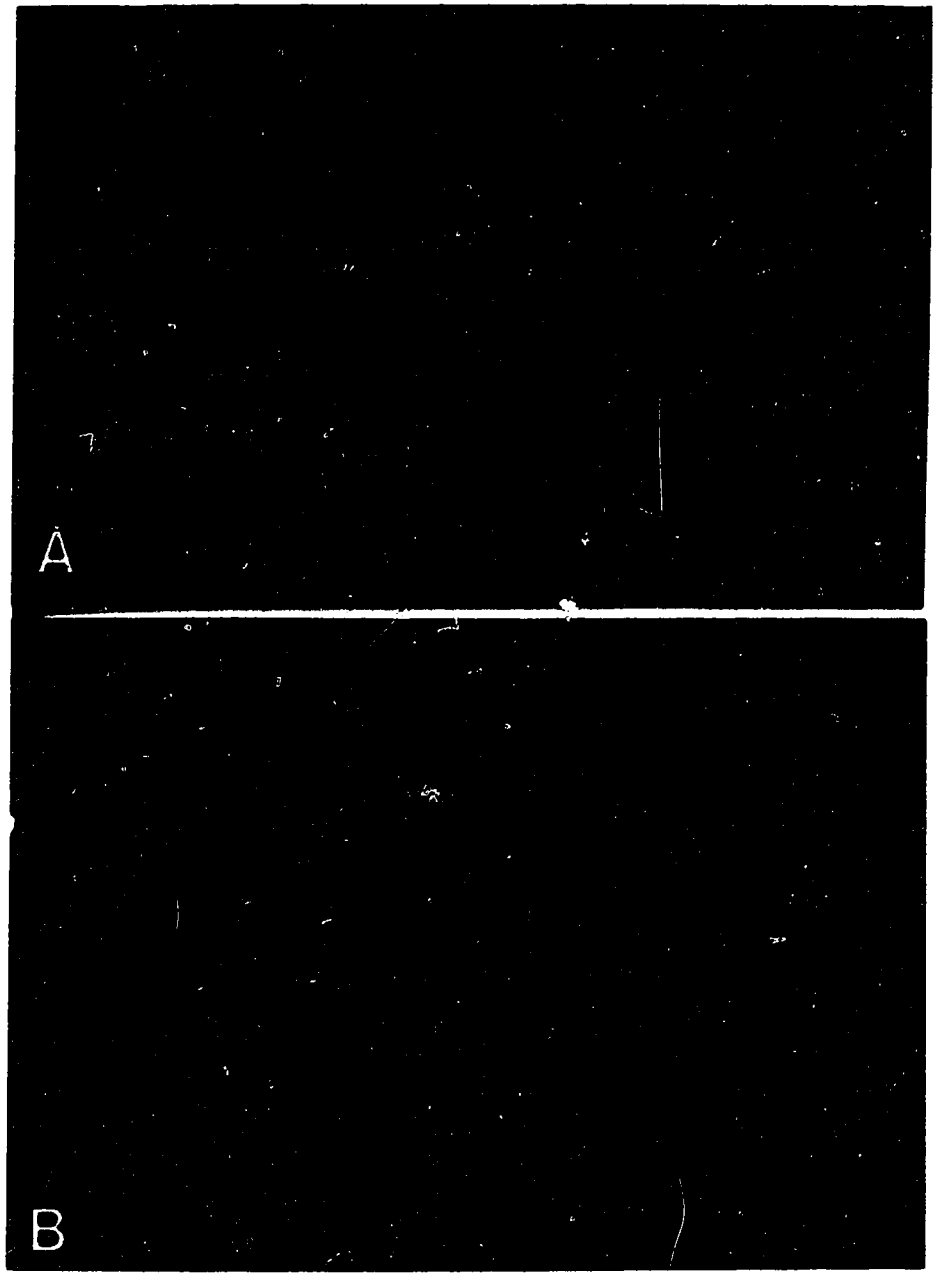

A
B

Fig. 2 The same as in Fig. III-1 except for confluent IEC-6 and MDCK monolayers. Confluent monolayers were obtained in the same way as described in the legend of Fig. III-1. Some of the peripheries in IEC-6 cells (A) were outlined by the ZO-1 labelling. Background fluorescence is also seen in the cell type. The labelling in MDCK cells (B) was much stronger than that in both IEC-6 and endothelial cells.

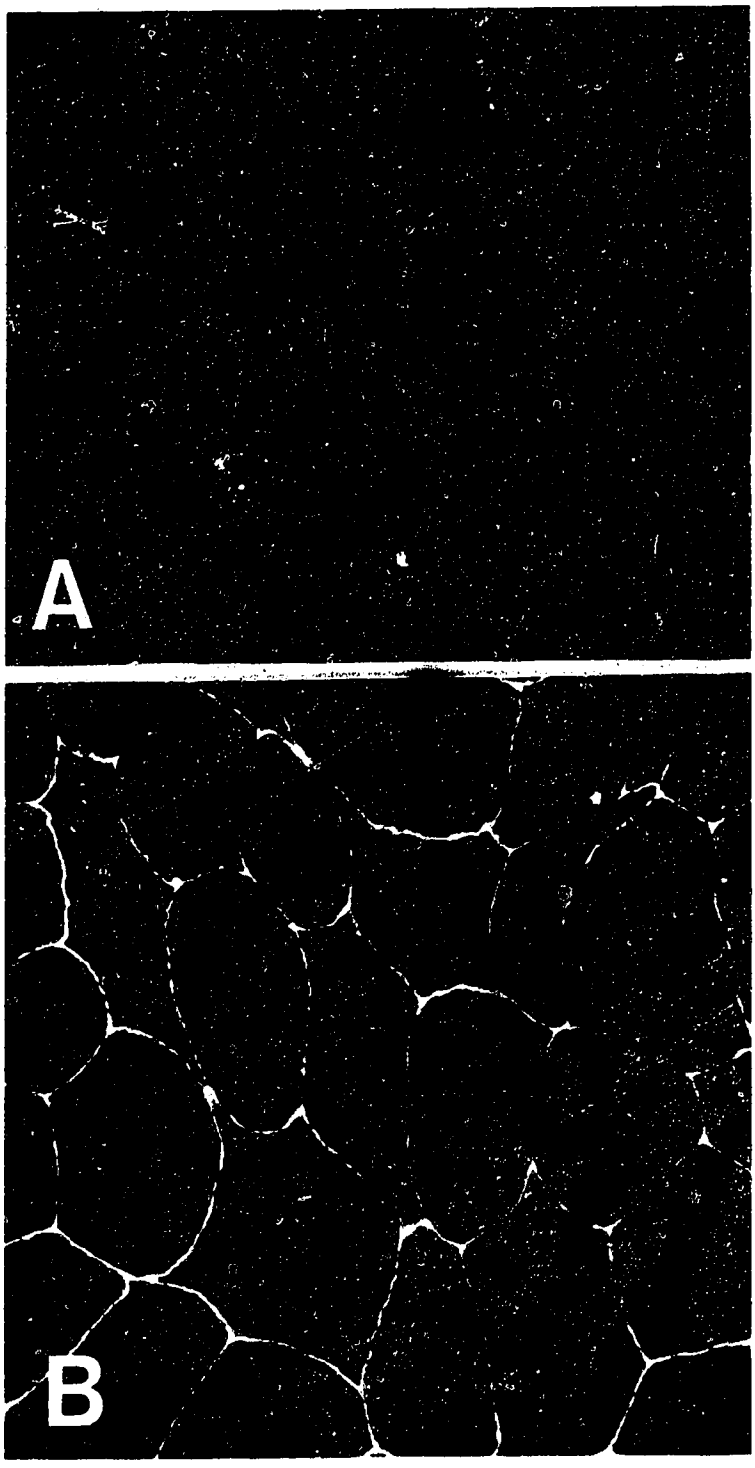
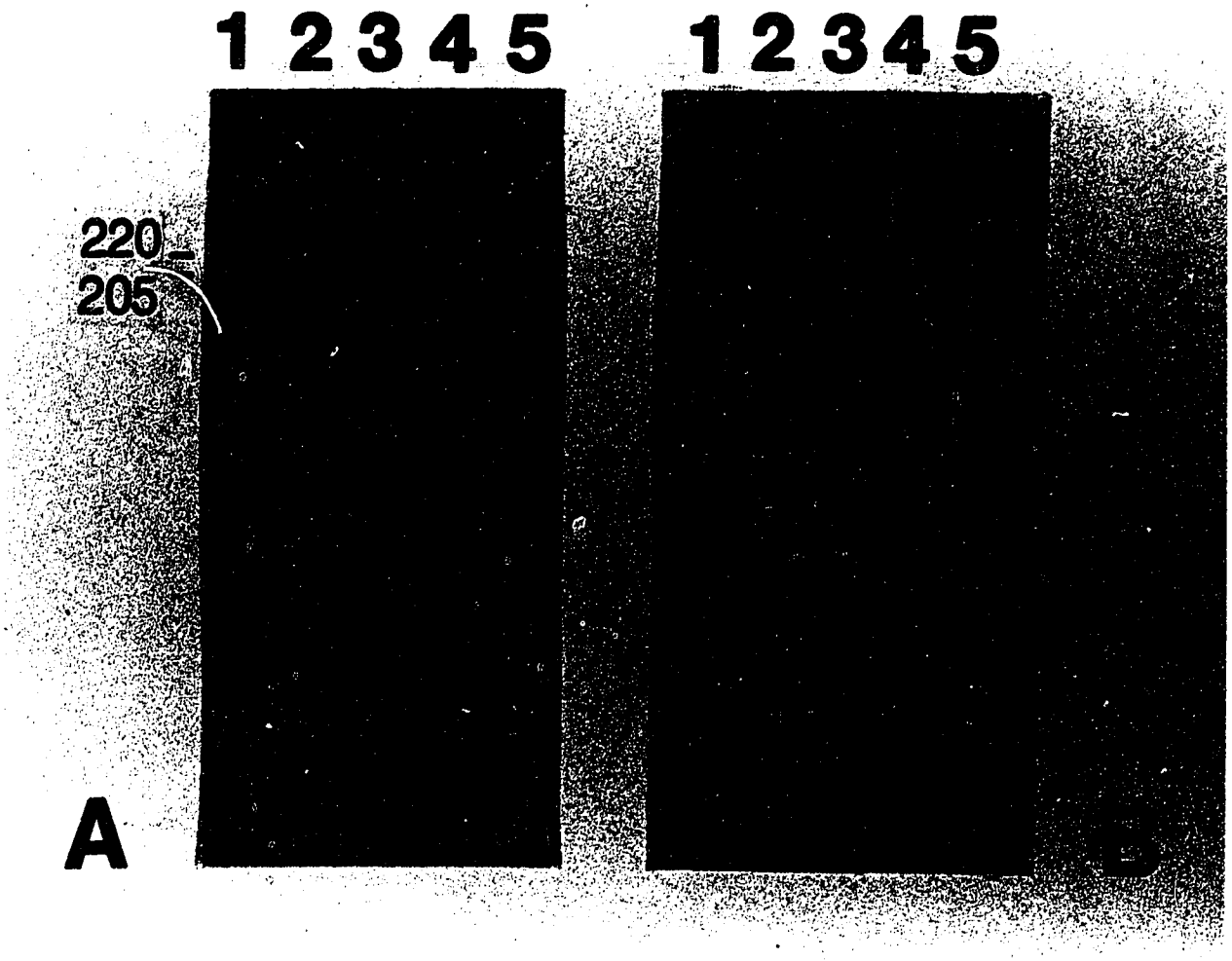

A
B

Fig. III-3 Endothelial monolayer confluency vs the content of the ZO-1 protein by immunoblotting. Cells were seeded at about 3% confluence (cells collected from 1 unit of surface area of confluent monolayer were seeded on to about 30 units of surface area) and grown for 3 (No. 1), 4 (No. 2), 5 (No. 3), 6 (No. 4) and 7 (no. 5) days. Numbers in A, B and C are corresponding. A, immunoblot result of the ZO-1 protein. B, Coomassie blue stained SDS gel. C, photomicrographs of cells of different confluencies. Urea extracts of the cells were used to run the SDS-PAGE. The same amount of protein was added to each sample. Microscopic magnification: 100 X; phase contrast.

A and B are on the opposing page , and C is on the page after.


1 2 3 4 5
1 2 3 4 5
220
205
A

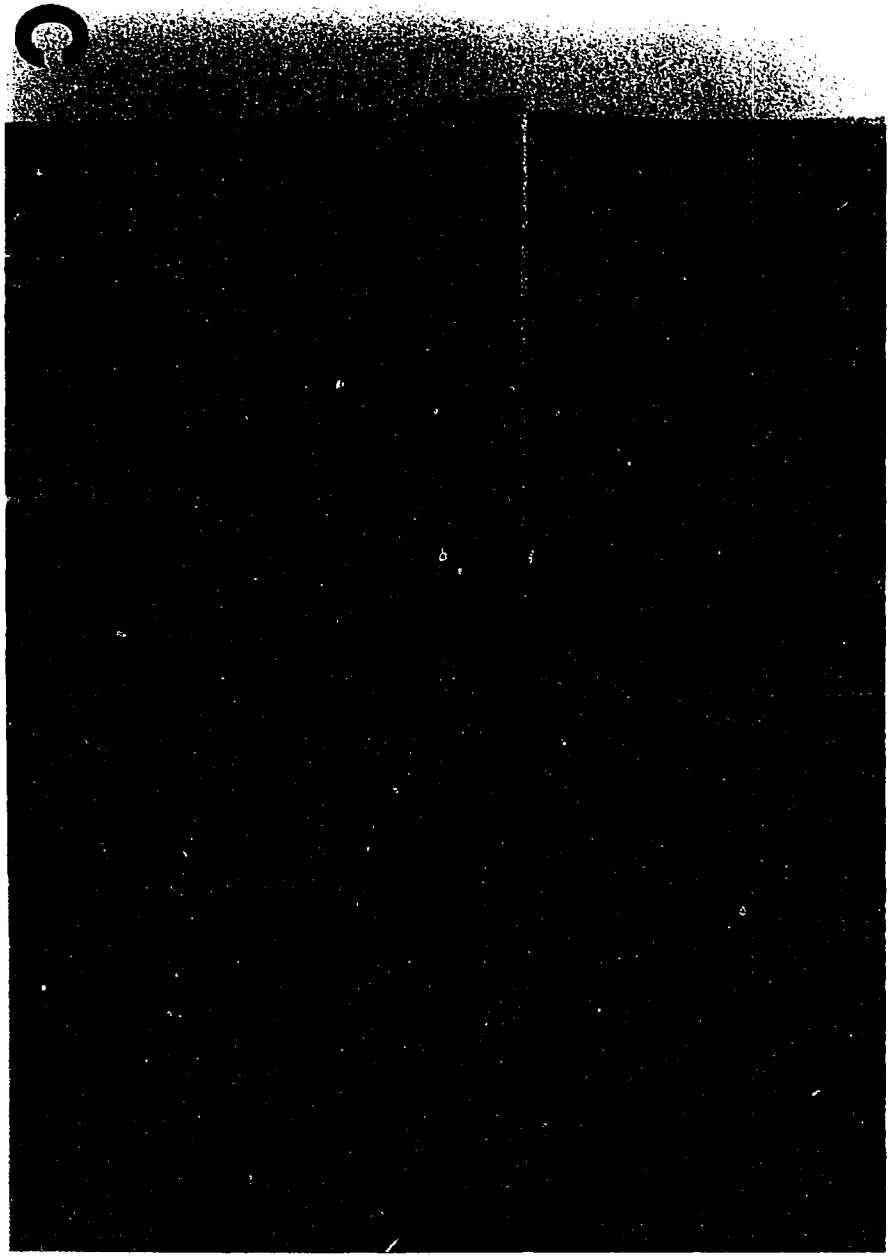


Fig. III-4 Endothelial monolayer confluency vs the expression of the ZO-1 protein by immunoblotting. Cells were seeded at about 100 % (lane 1 in A and B), 5 % (lane 2 in A) and 1.5 % (lane 3 in A and lane 2 in B) confluence and grown for the same length of time, i.e., 3 days. Panel A shows the immunoblot results from the SDS-PAGE of the urea extracts of the cells and B is the results from the SDS-PAGE of the whole cells. The samples were added to the gels based on protein in A, as in Fig. III-3 and Fig. III-5, and based on cell number in B (1.8 million cells for each lane). The numbers below the immunoblots are relative confluence when the cell monolayers were used. The monolayer of the largest cell number is designated as 100% confluence and the rest are relative to it. In both cases, a clear ZO-1 expression-confluency relationship is observed.

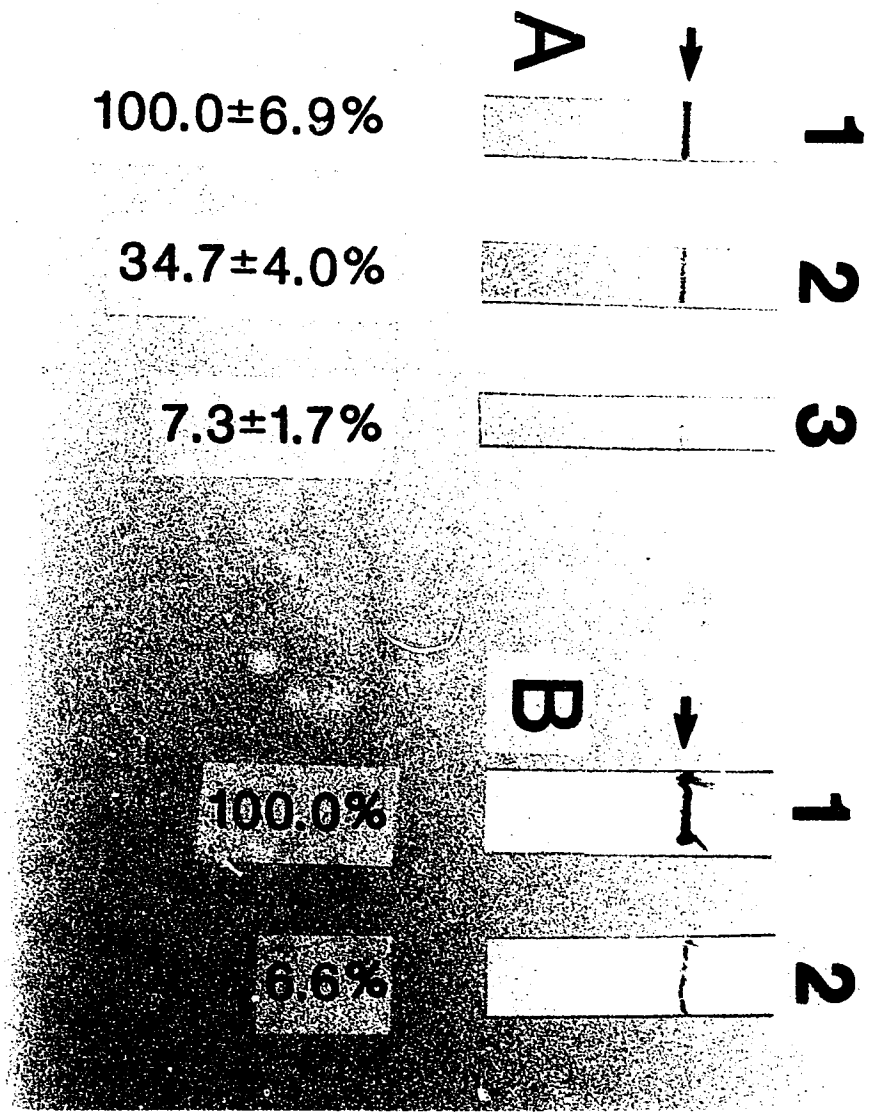

A
100.0±6.9%
1
34.7±4.0%
2
7.3±1.7%
3
B
100.0%
1
6.6%
2

Fig. III-5 Immunoblotting of the ZO-1 protein in MDCK (lane 1), IEC-6 (lane 2), endothelial (lane 3) and myeloma (lane 4) cells. Myeloma cells were grown in suspensions, whereas the rest were grown in monolayers. Cells were seeded so that they would become confluent for the monolayer-forming cells, and reach steady-state growth for myeloma cells. A, immunoblot transferred from a gradient (4.5%-18%) SDS gel. B is the immunoblot transferred from a nongradient gel (7%) which is for a more precise estimation of the molecular weight of ZO-1. The same amount of protein was added to each lane of the same SDS gel.


1 2 3 4
1 2 3
220
205
A
B

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CHAPTER IV

EFFECT OF PROTEIN KINASE C ON TRANSEPITHELIAL RESISTANCE AND CELLULAR DISTRIBUTION OF ZO-1 PROTEIN IN MADIN-DARBY CANINE KIDNEY CELL MONOLAYERS

KEY WORDS:

protein kinase C

transepithelial resistance

Madin-Darby canine kidney

tight junction

ZO-1 protein

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INTRODUCTION

The importance of protein kinase C in regulating cell functions has been well established. Physiologically, the enzyme is activated by a variety of receptor ligands through the hydrolysis of phosphatidylinositol 4,5-biphosphate (Berridge and Irvine, 1984). It is also activated directly by some phorbol esters (Castagna *et al.*, 1982, Kikkawa *et al.*, 1983), including phorbol 12-myristate 13-acetate (PMA) in the presence of calcium ion. Several protein kinase inhibitors have been reported, of which 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) is most potent and selective, although not specific, for protein kinase C (Nishikawa *et al.*, 1986). Protein kinase C is a widely distributed enzyme in mammalian tissues. Its has been demonstrated in Madin-Darby canine kidney (MDCK) cell line (Slivka *et al.*, 1986, Friedlander and Amiel, 1987) and in primary culture of renal tubular cells (Friedlander and Amiel, 1989).

High transepithelial resistance (TER) can develop across the confluent MDCK monolayer due to the presence of intercellular tight junctions (Cereijido *et al.*, 1978). Thus, change in tight junction or paracellular permeability can be assessed by TER measurement. Tight junction is a very dynamic structure and is subject to regulation by many factors, including calcium ion concentration (Martinez-Palomo *et al.*, 1980), cyclic AMP (Duffey *et al.*, 1981), temperature (Gonzalez-Mariscal *et al.*, 1984), osmolarity (Cereijido *et al.*, 1978), cytoskeleton-active agents (Bentzel *et al.*, 1980) and transportable nutrients glucose and amino acids (demonstrated in intestinal epithelium,

Pappenheimer, 1987). The effect of protein kinase C activation on tight junction permeability in MDCK monolayers has been described before (Ojakian, 1981). We attempted to further characterize the effect by examining the influence of protein kinase C on cellular distribution of the tight junction-associated protein ZO-1.

The ZO-1, well characterized in epithelial and endothelial cells (Stevenson *et al.*, 1986, Anderson *et al.*, 1988, Stevenson *et al.*, 1988), is exclusively localized to the tight junctions in MDCK cells (Stevenson *et al.*, 1986). There are changes in cellular distribution of the protein when cell-cell contact is disrupted by calcium ion depletion (Stevenson *et al.*, 1988). Therefore the ZO-1 protein is a good indicator of the change in tight junction integrity. We demonstrated that protein kinase C activation caused dramatic reduction and disruption of immunofluorescence labelling of the protein in cell peripheries while increasing tight junction permeability.

MATERIALS AND METHODS

CELL CULTURE

MDCK cells were purchased from American Type Culture Collection. Stock cells were maintained at 37°C in 75 cm^2 flasks (Becton Dickinson and CO.) in an incubator filled with 95% air, 5% CO_2. The medium used was minimum essential medium (MEM) supplemented with 10% bovine serum (FCS) and 1% penicillin-streptomycin (Sigma; final concentrations: penicillin 100 IU/ml and streptomycin 100 μg/ml). Cells were passaged by trypsinizing with Puck's saline containing 0.04% trypsin (Sigma) and 0.2 g/L disodium ethylenediamine tetraacetate (EDTA, Fisher) and splitting at a ratio of 1:5. All media were obtained from GIBCO.

For the purpose of measuring TER, MDCK monolayers were grown on Nucell culture inserts (24.5 mm diameter and 0.4 μm pore size; Nucleopore). The inserts were coated with fibronectin (used as a solution of 100 μg/ml; Sigma). Cells were trypsinized off the stock flasks as stated above and washed by centrifuging in a mixture of Dulbecco's modified Eagle's medium (DMEM) and medium F-12 (50%/50%; both from GIBCO). They were seeded at a density of 1 x 10^6 cells/cm^2 on the inserts. 3 ml and 4 ml of DMEM/F-12 with 10% FCS and 1% penicillin-streptomycin were added to the inside and outside of each insert, respectively. Monolayers were allowed to grow for 24 hours before use unless otherwise specified.

TER MEASUREMENT

The TER of MDCK monolayers was measured using a Millicell-ERS apparatus (Millipore). This apparatus has two probes, each of which contains a current-applying electrode and a voltage-measuring electrode. The probes were mounted on an adjustable stand and introduced to the incubator where monolayer TER was to be measured. The incubator atmosphere was the same as that normally used for cell culture unless otherwise specified. For the measurement, the two probes were positioned such that one was inside and the other was outside of the inserts. The recording part of the unit was located outside of the incubator. TER generated by the cell monolayer was obtained by subtracting the background value of filter insert alone from the total value. All the TER measurements were carried out in DMEM/F-12 (50%/50%) in the presence of 10% FCS unless otherwise specified.

IMMUNOFLUORESCENCE LABELLING OF ZO-1

Labelling of the tight junction protein ZO-1 was carried out on monolayers grown on Lab-Tek chamber/slides (NUNC Inc.). The slides were coated with 100 μg/ml polylysine (Sigma) in Dulbecco's phosphate buffered saline (PBS; GIBCO) and washed with FCS-free DMEM/F12 before cells were seeded. Confluent MDCK monolayers were formed by plating cells at a density of 1 x 10^6 cells/cm^2 and allowing to grow for 24 hours in DMEM/F12 with 10% FCS and 1% penicillin-streptomycin. Labelling of the ZO-1 protein was carried out on MDCK cells permeabilized with 0.1% Triton X-100 as described previously (Stevenson *et al.*, 1986) using the anti-ZO-1 monoclonal antibody R40.76 followed by biotinylated rabbit anti-rat IgG (Vector; 7.5 μg/ml) and then Texas red-conjugated streptavidin (Amersham, 4 μl/ml). Slides were mounted in the mountant made of Mowiol 4-88 (Calbiochem) (Heimer & Taylor, 1974, Osborn & Weber, 1982).

RESULTS

PROTEIN KINASE C AND TER GENERATION

As previously described (Cereijido *et al.*, 1978), the development of TER across MDCK cell monolayers increased slowly over the first 8 to 12 hours and rapidly in later hours during a 24 hour period of incubation (Fig. IV-1). At the seeding density of 1 x 10^6 cells/cm^2 morphologically confluent monolayers formed once the cells had settled on the filters within 3 hours as examined by phase contrast microscopy. The time delay between the formation of morphologically confluent monolayers and the development of high TER probably was due to the need of the cells in monolayer to align properly with one another. PMA added at a final

concentration of 40 ng/ml dramatically blocked the TER development (Fig. IV-1). In other words, protein kinase C prevented the formation of the tight junctions.

PROTEIN KINASE C AND PREFORMED TIGHT JUNCTIONS

The effect of protein kinase C activation on preformed tight junctions of MDCK cells is shown in Fig. IV-2 and Fig. IV-3. Cells were allowed to grow for 24 hours in order to have high monolayer TER or well-developed tight junctions. PMA caused a rapid decrease in TER with a half-time around 20 minutes (Fig. IV-2, A). This effect is temperature-dependent as PMA caused no decrease in TER at 4°C (Fig. IV-2, C). The effect of PMA was diminished in the presence of protein kinase C inhibitor H7 (Fig. IV-2, B). H7 added alone caused an increase in TER (Fig. IV-3) and the time course of this increase in resistance by H7 was more rapid than the decrease observed with PMA, with a half-time around 2 minutes. These indicate that protein kinase C has an effect in disrupting the preformed tight junctions. That inhibition of protein kinase C by H7 could induce a TER increase suggests that protein kinase C exerts its TER-decreasing effect under normal conditions.

PROTEIN KINASE C AND REFORMATION OF TIGHT JUNCTIONS

Fig. IV-4 shows the effect of PMA on the reformation of tight junctions following an opening of the paracellular pathway by calcium ion depletion. Treatment of MDCK monolayers with 2.5 mM EGTA in Ca^{2+}-free PBS for 2 minutes results in a partial openning of the tight junctions (Martinez-Palomo *et al.*, 1980), which allows for a faster recovery of

monolayer TER upon calcium ion replacement compared to complete openning of the structure with longer EGTA treatment. In the control (-PMA), recovery started in about 10 minutes and complete recovery was seen in about 1 hour. Presence of PMA (+PMA) essentially prevented the recovery of the monolayer TER. The initial small recovery was probably due to insufficient activation of protein kinase C at the beginning.

PROTEIN KINASE C AND ZO-1 PROTEIN DISTRIBUTION

Upon PMA treatment, confluent MDCK monolayers underwent dramatic morphological changes. As determined by phase contrast microscopy intercellular spaces became wider (Fig. IV-5, B), indicating a change in tight junction configuration. We compared a PMA-treated monolayer with a calcium ion depleted one (Fig. IV-5, C). There is a noticeable difference in morphology between the two. PMA caused a uniform change, whereas calcium ion depletion affected some parts of the monolayer more than the others. Unlike calcium ion depletion, PMA did not affect cell adherence to the substratum even with prolonged incubation. Even with these differences both caused widening of intercellular spaces. When a confluent MDCK monolayer was treated with PMA, the distribution of the tight junction-associated protein ZO-1 by immunofluorescence labelling underwent dramatic changes. Normally the protein is exclusively localized to junctional areas as shown in Fig. IV-6, A. PMA caused the protein to diffuse out of junctional areas (Fig. IV6, B). Not only was there reduction but also disruption of the labelling in the junctional regions.

DISCUSSION

We demonstrated that the protein kinase C activator PMA prevented the formation and reformation of MDCK tight junctions and increased the permeability of preformed tight junctions. The overall effect of protein kinase C on the tight junctions is to keep them open and to make the epithelial monolayer more permeable. This effect of protein kinase C was demonstrated at 40 ng/ml of PMA, a very low concentration compared to those used in other protein kinase C studies (Friedlander and Amiel, 1989, Portilla *et al.*, 1988), suggesting that protein kinase C is a sensitive modulator of TER or tight junction permeability in MDCK monolayers. Under normal conditions (or unstimulated state) the tight junctions may be subject to constant influence by the kinase since inhibition of the enzyme by H7 (Fig. IV-2 and Fig. IV-3) could increase monolayer TER. In light of the fact that cAMP could cause the same TER changes as H7 did in MDCK (unpublished results) and in amphibian *Necturus* gallbladder (Duffey *et al.*, 1981) epithelial cells, the possibility that H7 might have worked through cAMP-dependent protein kinase is ruled out even though H7 is also an inhibitor of cAMP-dependent kinase (Hidaka *et al.*, 1984).

The effect of protein kinase C on the TER in MDCK (Ojakian, 1981) and in another kidney tubular cell line LLC-PK_1 (Mullin and O'Brien, 1986) has been demonstrated by using another phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA). Our results confirmed it and also extendeded it by the demonstration of the novel influence of protein kinase C on the cellular distribution of the protein ZO-1 and the data using protein kinase C inhibitor.

PMA caused a dramatic reduction and disruptions of ZO-1 immunofluorescence labelling in junctional areas (Fig. IV-6). There was a redistribution of the protein from cell peripheries to cytoplasm. Stevenson *et al.* (1988), and Siliciano and Goodenough (1988) have shown that calcium ion depletion caused a similar reduction and disruption of ZO-1 labelling in cell periphery and redistribution of the protein to cytoplasm in MDCK cells. These data imply that once cells lose contact with one another or the tight junctions are disrupted, the tight junction protein ZO-1 will lose association with cell periphery. Protein kinase C activator TPA was also shown to cause diffusion of immunofluorescence labelling of the peripheral actin band (Kellie *et al.*, 1985) which is also considered to be tight junction-associated.

The protein ZO-1 has been demonstrated to be phosphorylated on serine residues (Anderson *et al.*, 1988) even though we are not sure whether the phosphorylation is stimulated by protein kinase C. It could well be that the increase in tight junction permeability was mediated by increased phosphorylation of the ZO-1 induced by protein kinase C. It is implied by the study by Stevenson and co-workers (1989). They found that two strains of MDCK cells, one generating a TER of 4350 ± 1864 ohms.cm^2 and the other 55 ± 29 ohms.cm^2, differ in the phosphorylation of their ZO-1 by a factor of 2 with the protein in the lower resistance strain having more phosphorylation.

The mechanism by which protein kinase C activation caused such changes is not known at this stage. The kinase might increase tight junction permeability by acting on ZO-1 protein or/and other tight junction

proteins. Our results and the data on ZO-1 phosphorylation in two MDCK strains differring in TER (Stevenson *et al.*, 1989) suggest this. Or protein kinase C might permeabilize the tight junctions by acting on the cytoskeleton. The results of TPA-induced changes in the immunofluoresence labelling of actin (Kellie *et al.*, 1985) may give support to this. According to the tight junction model by Madara (1988, 1989), cytoskeleton (actin) is an integrated part of tight junction and plays a vital role in the regulation of tight junction permeability.

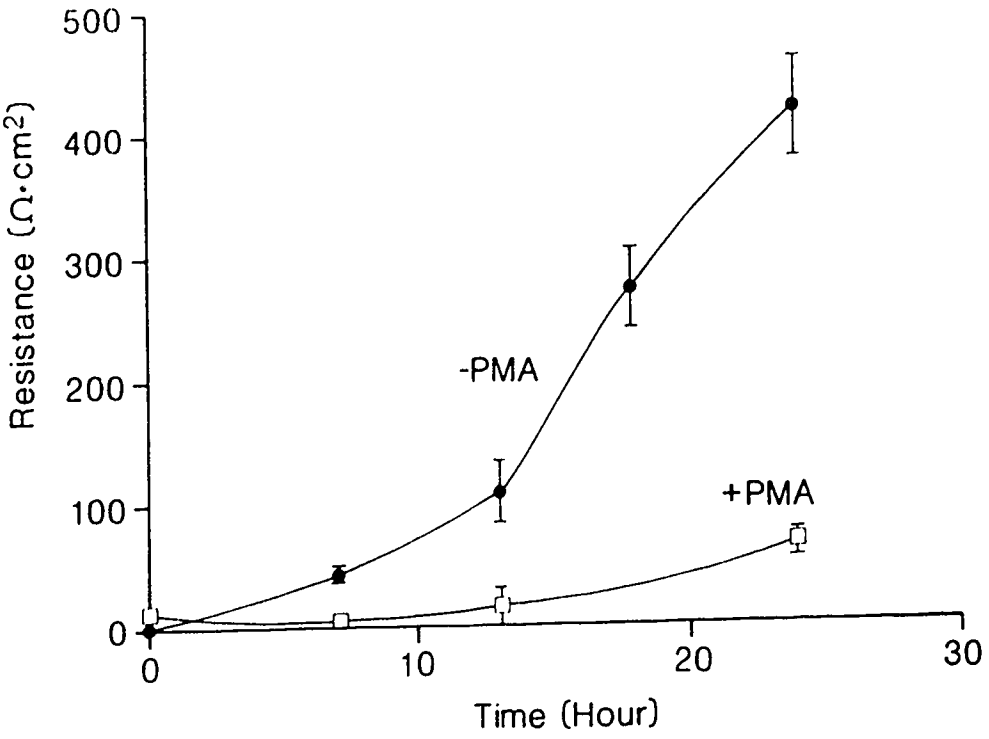

Fig. IV-1 Effect of PMA on the generation of TER in MDCK monolayers. Cells were seeded onto Nucleopore culture inserts at a density as stated above. PMA which was dissolved in dimethyl sulfoxide (DMSO) was added to the culture medium at a final concentration of 40 ng/ml at the time of seeding (+PMA). The same amount of DMSO was added to the control (-PMA). Serial TER measurements were carried out under sterile conditions. Cells were maintained under the same conditions as regular cultures. Each point represents the average of the values from 3 samples.

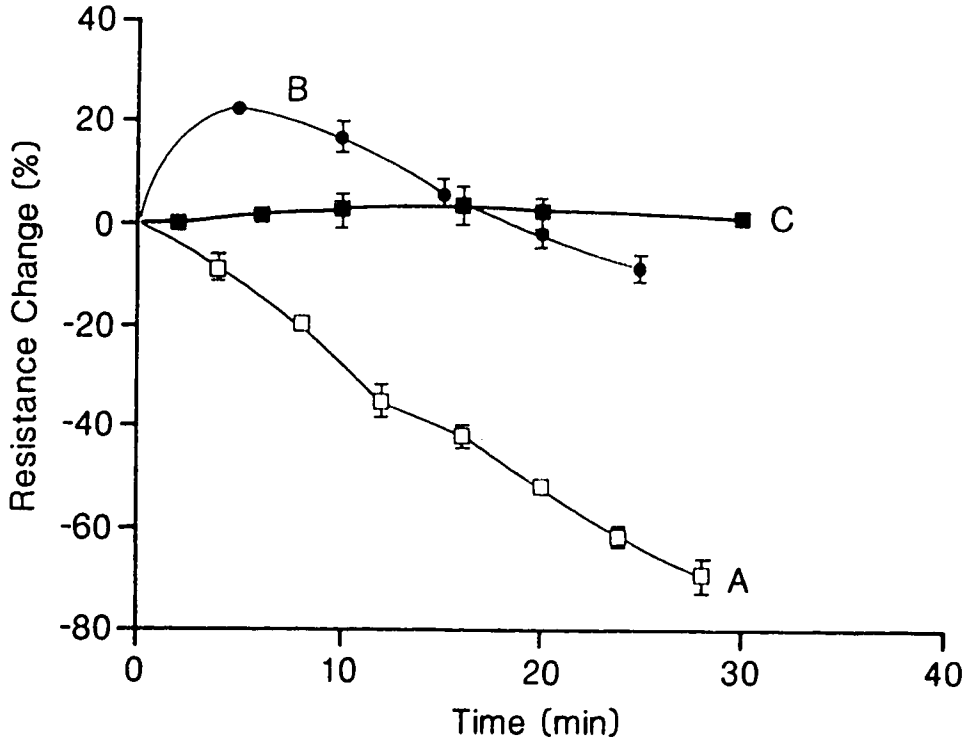

Fig. IV-2 Effect of PMA on TER in confluent MDCK monolayers. Confluent monolayers formed after growth for 24 hours. In curve A, PMA at a final concentration of 40 ng/ml was added. Curve B represents the addition of protein kinase inhibitor H7 at a final concentration of 20 μM in addition to PMA of the same concentration as in curve A. The two reagents were added simultaneously. Curve C shows the effect of PMA on TER at low temperature, i.e. at 4°C instead of 37°C, as in curves A and B. The same concentration of PMA was used in the latter case. Control values as shown in Fig. IV-3 have been subtracted. On the ordinate, TER changes are expressed in percentage increase or decrease compared to the starting TER levels. The data was processed as follows: percentage change for an individual sample was first calculated against the corresponding starting TER value, then the average of these changes in percentage from 2 samples was obtained in order to minimize the variation created by the difference in the starting TER levels.

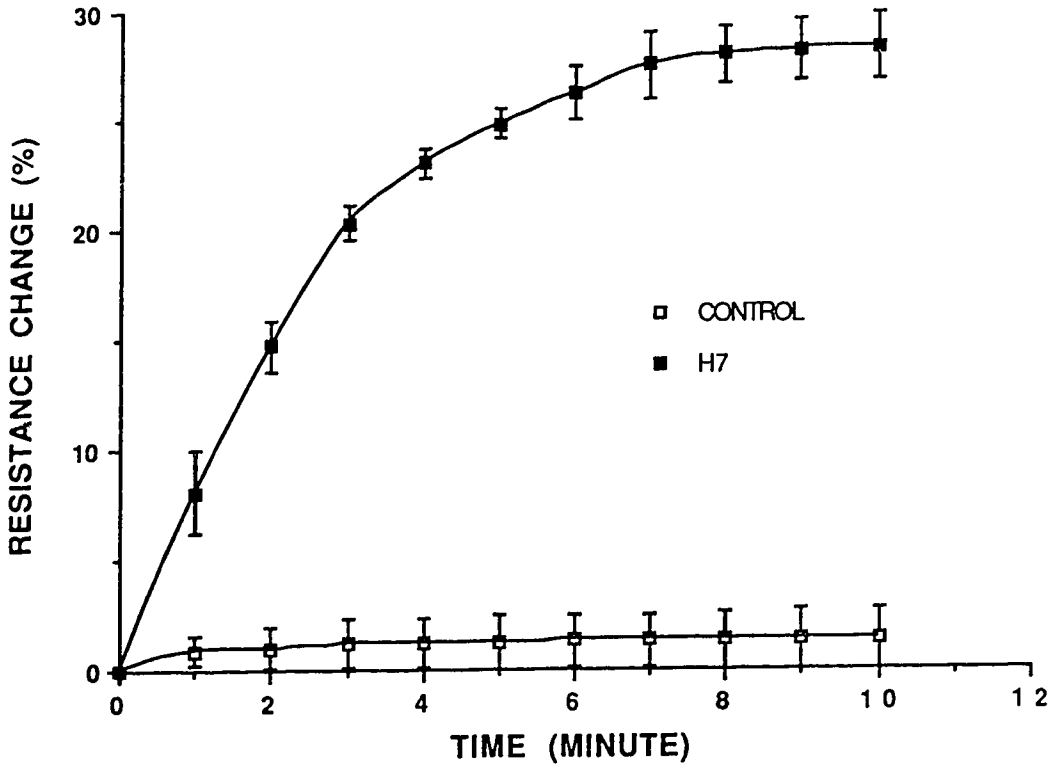

Fig. IV-3 Effect of protein kinase inhibitor H7 on the TER in confluent MDCK monolayers. Cells were grown for 24 hours. H7, which was dissolved in a small volume of distilled water, was added at a final concentration of 20 μM. To the control, only the solvent of the same amount was added. Continuous TER measurements were carried out for each sample. n=4 for H7 and 2 for control.

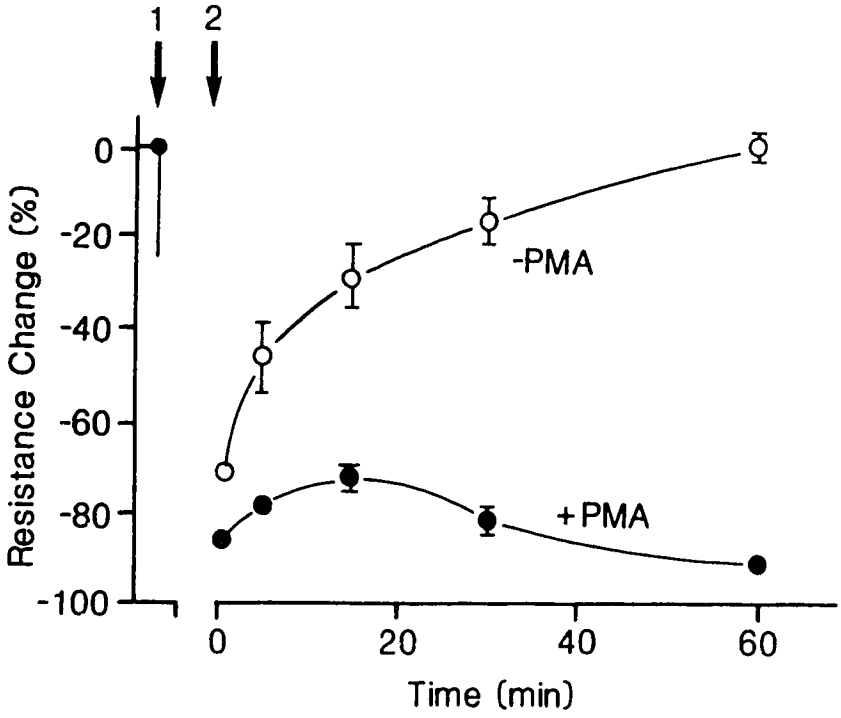

Fig. IV-4 Effect of PMA on the recovery of TER after calcium ion depletion in confluent MDCK monolayers. Medium DMEM/F-12 (50%/50%) plus 10% FCS used to grow the cells was changed to calcium-free PBS containing 2.5 mM ethylene glycol bis-(beta-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA from Sigma). Arrow 1 indicates this medium change. The calcium-free EGTA buffer was allowed to remain for 2 minutes. Calcium depletion was terminated by removing the calcium-free EGTA buffer and washing the cell monolayers once with PBS (calcium-free without EGTA). Calcium ion-containing DMEM/F-12 plus 10% FCS was then added, indicated by arrow 2. PMA was added to the samples indicated. The calcium concentration in the medium was 1.8 mM without FCS and close to or slightly higher than this value in the presence of 10% FCS. n=3.

Fig. IV-5 Effect of PMA on the morphology of confluent MDCK monolayer. Cells were grown on petri dishes (60 x 15 mm, Becton Dickinson and Company,) to confluence in DMEM/F-12 plus 10% FCS. Cells shown in A were those not treated. B represents the cells treated with PMA at a final concentration of 40 ng/ml for 1 hour. C shows the cells treated with the calcium ion-free EGTA buffer mentioned in Fig. IV-4 for 5 to 10 minutes.

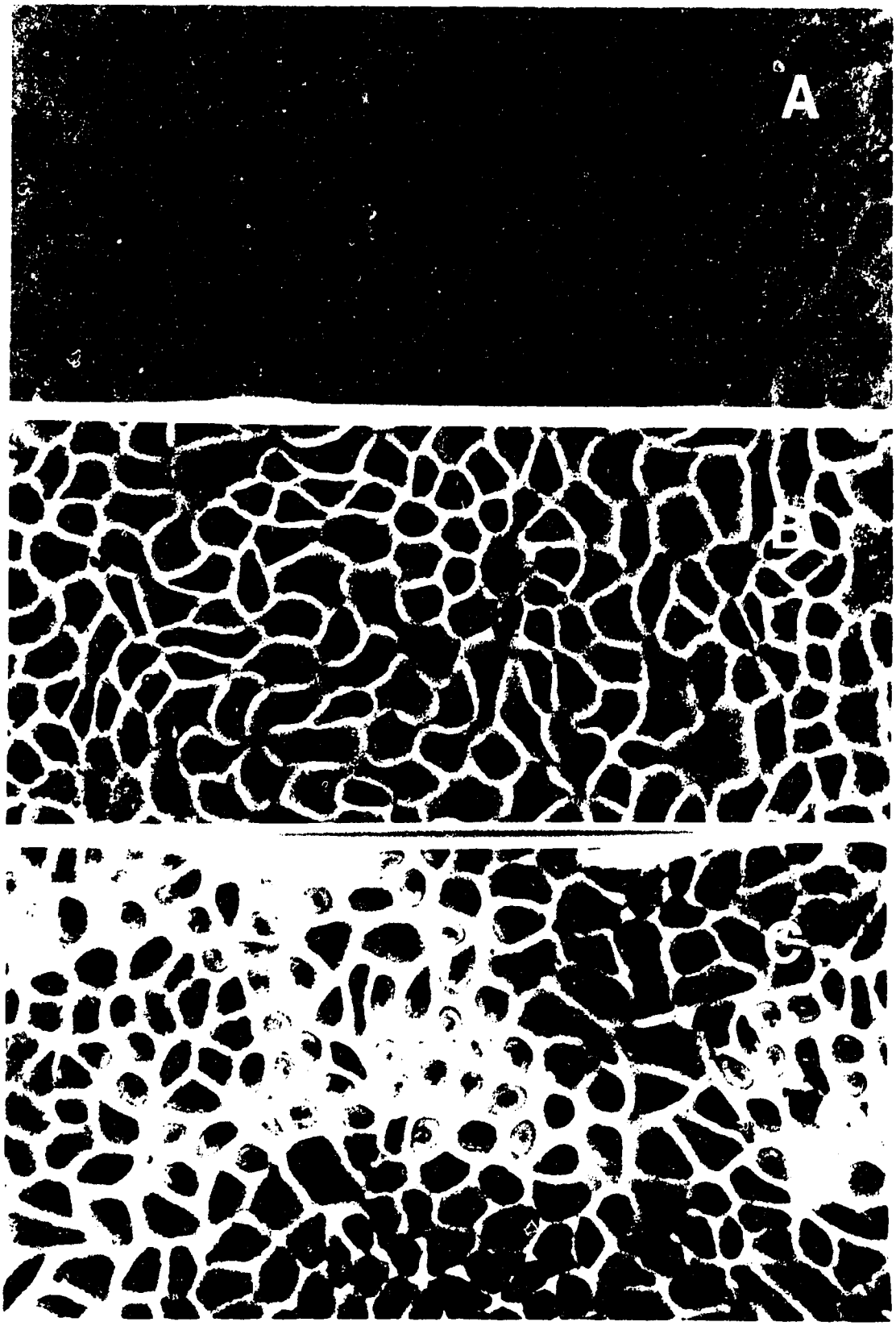
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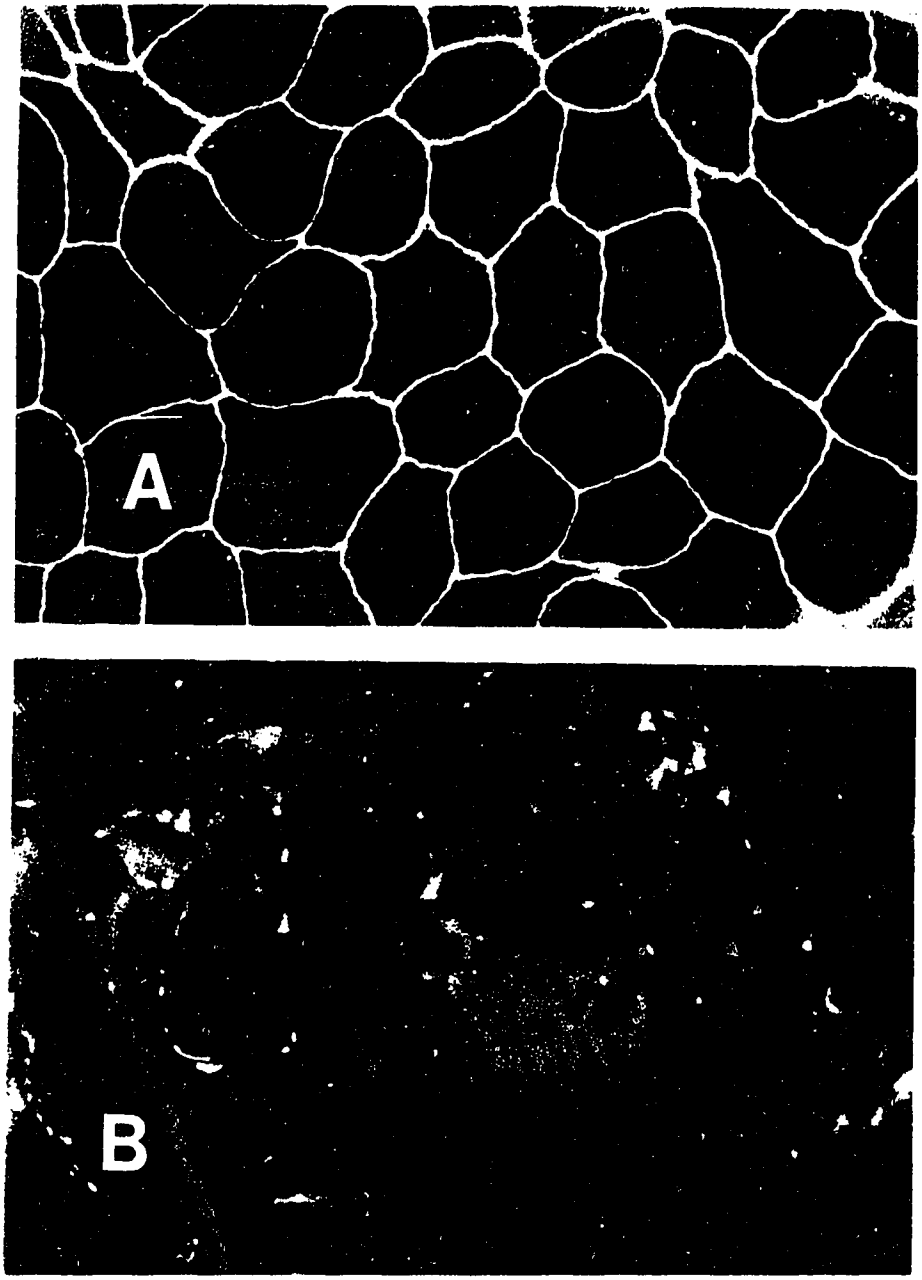
Fig. IV-6 Effect of PMA on the distribution of tight junction protein ZO-1 in confluent MDCK monolayers. Cells were grown on Lab-Tek 8-chamber/slides for 24 hours to form confluent monolayers. The seeding density was similar to the cultures on the filter inserts. See Materials and Methods for procedural details of immunofluorescence labelling. Panel A shows the labelling of the protein ZO-1 of the cells untreated whereas the labelling of the treated cells is shown in B. PMA at a final concentration of 40 ng/ml was added to the cells for 1 hour before the labelling.


A
B

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CHAPTER V

EFFECTS OF cAMP ON TRANSEPITHELIAL RESISTANCE IN MADIN-DARBY CANINE KIDNEY MONOLAYERS

KEY WORDS:

Madin-Darby canine kidney

cAMP

transepithelial resistance

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INTRODUCTION

The Madin-Darby canine kidney (MDCK) cell is a cell line originally established from canine kidney tubular cells (1). By formation of tight junctions between neighboring cells, confluent monolayers of these cells can develop relatively high transepithelial resistance (TER). Thus, the value of TER reflects the tightness of the tight junctions. The MDCK cells are among the most commonly used cells for studying the properties of the tight junctions (2, 3, 4). The tight junction is a very dynamic structure and its permeability properties have been shown to be regulated by many factors, some of which are of physiological significance. For instance, calcium ion is essential for maintaining the intact tight junction structure (3). Protein kinase C activation decreases TER in the monolayers of MDCK (5) and another kidney tubular cell line LLC-PK_1 (6). Glucose and amino acids can make tight junctions more permeable in intestinal epithelia (7). Cytoskeleton-active agents, e.g., cytochalasins, have been shown to affect TER of epithelial monolayers (8, 9). In addition, physical factors such as temperature (2) and osmolarity (2) also affect tight junction permeability.

Like many other types of cells, MDCK cells have been shown to have an active cAMP/adenylate cyclase system (10). cAMP analogues have been shown to decrease the permeability of the tight junctions in amphibian *Necturus* gallbladder epithelium, which occurs within an hour after the drugs were added (11). In this study we attempted to characterize this short-term effect of cAMP on the tight junctions of the mammalian epithelial line MDCK cells and to examine as well what if any long-term

effects of cAMP on the structure might be observed which were distinct from the short-term one.

MATERIALS AND METHODS

CELL CULTURE

The MDCK cells were purchased from American Type Culture Collection. Cells from 59 to 68 passages were used in these experiments. Stock cells were maintained at 37°C in 75 cm^2 flasks (Becton Dickinson and Company) under an atmosphere of 95% air and 5% CO_2. The medium used to maintain stock cultures was minimum essential medium (MEM) supplemented with 10% bovine serum (FCS; GIBCO) and 1% penicillin-streptomycin (Sigma; final concentrations: penicillin 100 IU/ml and streptomycin 100 μg/ml). All media were obtained from GIBCO. Stock cells were passaged by trypsinizing with Puck's saline containing 0.04% trypsin (Sigma) and 0.2 g/L disodium ethylenediamine-tetraacetate (EDTA; Fisher) and splitting at a ratio of 1:5.

For the purpose of measuring TER, monolayers were prepared on Nucell culture inserts (24.5 mm diameter and 0.4 μm pore size; Nucleopore). The inserts were coated with bovine fibronectin (Sigma) by leaving the phosphate buffered saline (PBS; GIBCO) containing 100 μg/ml of fibronectin on the inserts for 5 to 10 min, then aspirating off and letting them dry. They were contained in six-well tissue culture plates (Costar). Cells were trypsinized off the stock flasks as stated above and washed by centrifuging in a mixture of Dulbecco's modified Eagle's medium (DMEM) and medium F-12 (50%/50%; both from GIBCO). Cells were seeded at 1 x 10^6 cells/cm^2 onto the inserts. Once settled at this high

density, they were able to form a confluent monolayer with high TER in 24 hours. 3 ml and 4 ml of DMEM/F-12 with 10% FCS and 1% penicillin-streptomycin as aforementioned were added to the inside and outside of each inserts, respectively. Monolayers were allowed to grow under the atmosphere used for stock culture for 24 hours unless otherwise specified. There was no medium change to the cells other than the stock cultures.

TER MEASUREMENT

TER in MDCK monolayers was measured using a Millicell-ERS apparatus (Millipore). It consists of two probes and a recording unit. Each probe contains two electrodes, one for applying current and one for measuring voltage. Electrical resistance across the two probes can be read out from the LCD of the recording unit. The probes were mounted on an adjustable stand and introduced to the incubator where TER across monolayers was to be measured. The incubator atmosphere was the same as that normally used for the stock cell cultures. In making the measurements, two probes were positioned in such a way that one was inside and one was outside of the inserts with monolayer-containing filters forming the division between the probes. TER contributed by cell monolayer was obtained by subtracting the background electrical resistance value in the absence of cells from the total value. Only the net TER values will be shown. All the TER measurements were carried out in DMEM/F-12 (50%/50%) in the presence of 10% FCS.

^{3}H-THYMIDINE INCORPORATION, UPTAKE and CELL PROTEIN MEASUREMENT

Cells were collected by trypsinization as stated, and seeded onto the six-well culture plates (Costar) at the density shown in Table V-1 to form non-confluent monolayers. ^{3}H-thymidine (Du Pont) at a final concentration of 2 μCi/ml, and Bt2cAMP and cAMP-elevating agents at the concentrations indicated in the concerned legend were added at the time of seeding. ^{3}H-thymidine uptake is defined as the total radioactivity associated with cells; incorporation as the radioactivity precipitable with 15% trichloroacetic acid (TCA; Sigma). Incubation time for these assays was 24 hours. Cell protein measurement using the Lowry assay (12) was done at the same time as the incorporation and uptake, i.e. at the end of 24 hour incubation. These experiments were carried out in DMEM/F-12 (50%/50%) with 10% FCS.

^{14}C-LEUCINE INCORPORATION

Non-confluent MDCK monolayers were prepared as described in Fig. V-5. ^{14}C-leucine (Du Pont) at a final concentration of 0.25 μCi/ml was added to the cells at the time of seeding. Cells were allowed to grow for 24 hours. They were harvested using a rubber policeman into distilled water and disrupted with a cell disruptor (Branson Sonic Power Co.). Aliquots of the cell lysates were mixed with TCA to a final concentration of 15%. The precipitates were then scintillation-counted to determine the incorporation of ^{14}C-leucine into protein.

Bt2cAMP and cholera toxin were dissolved in DMEM/F-12, forskolin in dimethyl sulfoxide (DMSO), and papaverine and cycloheximide in 75% ethanol. Bt2cAMP, cholera toxin and forskolin solutions were sterilized, in case needed, by passing through filters of 0.22 μm pore size.

Papaverine and cycloheximide solutions were used without filtering for sterile purpose. The amount of solvents used was less than 0.1%; either DMSO or ethanol did not cause significant solvent-related electrical resistance change in the MDCK monolayers. Bt2cAMP, cholera toxin, forskolin and cycloheximide were purchased from Sigma, and papaverine from Aldrich.

The statistics were done using student t-test.

RESULTS

We have demonstrated that cAMP analogue Bt2cAMP and cAMP-elevating agents increased TER in confluent MDCK monolayers in short-term and decreased it with prolonged incubation. The latter effect could be blocked by cycloheximide.

SHORT-TERM EFFECT OF cAMP ON TER

We chose four agents in testing the effect of intracellular cAMP. Forskolin activates adenylate cyclase directly. Cholera toxin activates it through GTP-binding protein. Bt2cAMP is an analogue of cAMP. Papaverine raises intracellular cAMP level by inhibiting the degradation of cAMP by phosphodiesterase. As shown in Fig. V-1, Bt2cAMP increased monolayer TER in MDCK cells. In the presence of papaverine the TER increase was greater, as expected. The effect of Bt2cAMP at 2 hours represents maximum or near maximum response. Papaverine is a "dirty" drug to use, since it has other actions such as antagonizing intracellular calcium (13) besides raising intracellular cAMP (14). Therefore, we only consider the data from papaverine as supplementary.

We followed the time course of the response to forskolin up to 9 hours (Fig. V-2). Forskolin caused a rapid increase in TER which peaked around 1 to 2 hours after the drug was added. The TER started to drop towards the original level thereafter. Cholera toxin also caused TER to increase in short-term (Fig. V-3). Cholera toxin gave a slightly slower response than forskolin, which may be due to the fact that the activation pathway of adenylate cyclase is longer for cholera toxin than for forskolin. In short, the cAMP analogue and intracellular cAMP-elevating agents all had TER-increasing effect within a few hours.

LONG-TERM EFFECT OF cAMP ON TER

As shown in Fig. V-2, forskolin at 100 μM increased TER in MDCK monolayers by nearly 24% at 2 hours. Thereafter, the level started to decrease and reached close to control values in 9 hours. In contrast to the increase, the decrease in TER is a gradual, slow process. By prolonged incubation of confluent monolayers for 24 hours, Bt2cAMP, forskolin and cholera toxin all induced TER to drop significantly to below control level (Fig. V-4). We did not follow the TER changes in response to these agents continuously throughout the period of 24 hours, since maintaining sterility would be a problem with multiple measurements. Microscopically, there was no noticeable morphological change in the continuity of the cell monolayers associated with the prolonged incubation. Even though dome formation is known to occur in the confluent monolayer sitting on impermeable support (14, 15), we, however, did not find it to be the case in the monolayers grown on permeable filters.

cAMP AND CELL PROLIFERATION

Table V-1 shows the effect of Bt2cAMP, forskolin and cholera toxin on cell proliferation (or cell growth). Non-confluent MDCK monolayers, instead of confluent ones as used in the TER studies, were used in order to increase the sensitivity of the assay. At a seeding density of $1x10^5$ cells/cm^2, a monolayer of less than 50% confluence would form once the cells had settled on the plate surface. Thus room was allowed for the cells to proliferate. Bt2cAMP, forskolin and cholera toxin all had effect in stimulating the non-confluent cells to grow. In response to these agents, DNA synthesis which is reflected by ^{3}H-thymidine incorporation (Table V-1, A) was increased. As exemplified by Bt2cAMP, they caused a corresponding increase in cell mass which is indicated by cell protein measurement (Table V-1, B). Uptake of ^{3}H-thymidine by the cells was also increased (Table V-1, C).

TER AND CELL PROLIFERATION

We examined the effect of cycloheximide on Bt2cAMP-induced TER decreases in order to further test the hypothesis that cell proliferation may be the mediator of the long-term influence of cAMP on tight junction permeability. Cycloheximide is a protein synthesis inhibitor. Figure V-5 shows that it caused TER to increase in confluent MDCK monolayers and that the TER-decreasing effect of Bt2cAMP was totally blocked by cycloheximide. The TER-increasing phenomenon of cycloheximide had been noticed before (19) although the possibility that it may occur through the inhibition of cell proliferation was not postulated. ^{14}C-leucine and ^{3}H-thymidine incorporation studies, which were done as described in

Materials and Methods in non-confluent monolayers, indicated that cycloheximide at 4 μg/ml resulted in protein sythesis inhibition of 98.2% in the absence and 97.7% in the presence of Bt2cAMP (compared to the same control without Bt2cAMP), and DNA synthesis inhibition of 95.9% in the presence of Bt2cAMP over a 24 hour period of incubation. Obviously, the DNA synthesis inhibition is a consequence of the inhibition of protein synthesis. The data suggest that the long-term effect of cAMP on TER is very likely the consequence of cell proliferation it induced.

DISCUSSION

Our studies showed that cAMP has distinct short-term and long-term effects on TER or tight junction permeability in confluent MDCK monolayers. The short-term effect of cAMP on TER in MDCK cells is in good agreement with the demonstration by Duffey et al. (11) in amphibian *Necturus* gallbladder and Powell et al. (16) in rabbit ileum epithelial cells that cAMP can increase the TER in the cell types, which is seen within an hour. Bt2cAMP, forskolin and cholera toxin all caused TER increases in MDCK cells in short-term. Though not fully known, the mechanism for this short-term effect of cAMP on TER in all these epithelial cell types presumably is the same. Duffey et al. (11) demonstrated an increase in the number of tight junction fibril strands or the depth of tight junction belts in *Necturus* gallbladder epithelium in response to cAMP.

Distinct from the short-term effect of cAMP is the long-term one. Bt2cAMP, forskolin and cholera toxin all cause the TER to significantly

drop to below control levels over a 24 hour incubation (Fig. V-4). The long-term effect is not only in direction but also in time course dissimilar to the short-term one. It is a slow and gradual process. It is likely induced by a different mechanism. The rationale for examining the growth-stimulating effect of cAMP in MDCK cells is the notion that cells may have to alter the interaction with their neighbors or change tight junction permeability when they proliferate. cAMP was shown to strongly stimulate MDCK cells to proliferate in non-confluent monolayers (Table V-1) which is in agreement with previous demonstrations (17, 18). We did not use the cells in confluent monolayers in which the long-term effect of cAMP on TER was demonstrated to examine cell proliferation simply because such cells would have a limited area for growth and the sensitivity would be very low. cAMP might not cause a very significant increase in the proliferation of the confluent cells as it did in the non-confluent ones. However, it would very likely give the confluent cells the potential to grow. Cell proliferation or the potential to proliferate is probably the mediator of the long-term effect of cAMP in MDCK monolayers. The increases in TER and the inhibition of the TER-decreasing effect of cAMP by cycloheximide provide strong evidence for this possibility. It is reasonable to believe that altering the proliferation rate of the cells in confluent monolayer by any means can affect the monolayer TER and that proliferation possibly exerts influence on TER even in the confluent monolayers which are at quiescent steady-state .

It has been shown that protein synthesis inhibition significantly inhibits the formation of epithelial tight junctions. However, once the

tight junctions have formed they are no longer sensitive to the protein synthesis inhibition (19), suggesting that the protein or proteins participating in tight junction formation are stable or of low turnover rate. The inhibitory effect of cycloheximide on cAMP-induced TER decreaseswas demonstrated by taking this advantage. Probably the structure of the tight junction is more important than or as important as the composition in deciding the tight junction permeability.

The tight junction protein ZO-1 has been well characterized in MDCK cells (20, 21). It has been shown to undergo changes in cellular distribution by immunofluorescence labelling when tight junctions are disrupted or altered by calcium chelation (22, 23). We examined the cellular distribution of the protein upon the treatment with Bt2cAMP for 24 hours. There was no noticeable change (not shown), suggesting that the structural changes caused by Bt2cAMP were too small to be detected by this technique.

In summary, cAMP decreased tight junction permeability in MDCK cells in short-term and increased it in long-term. The long-term effect is likely due to increased cell proliferation.

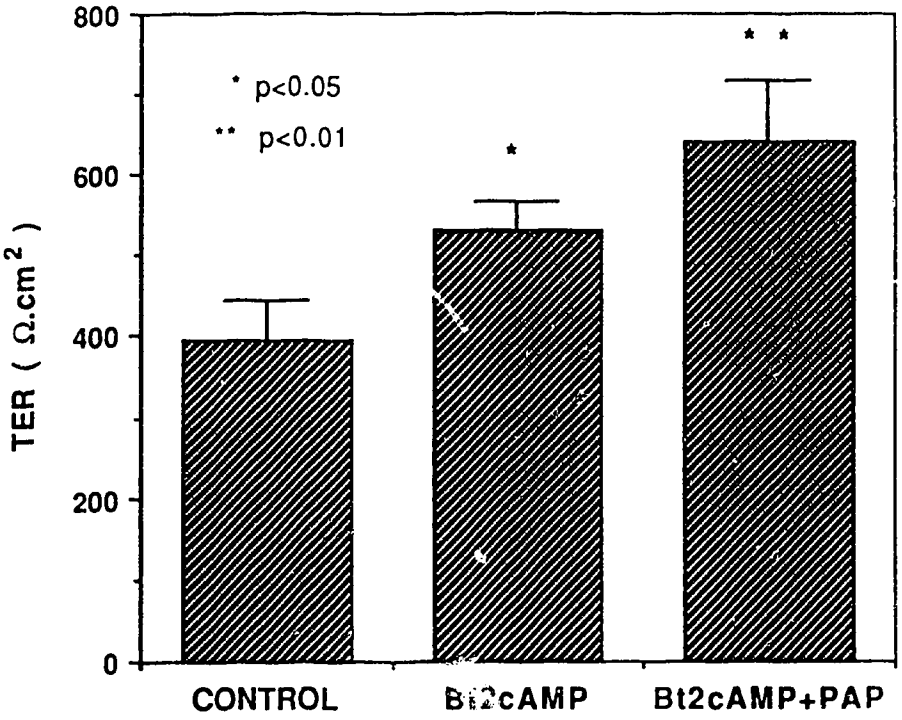

Fig. V-1 Effect of Bt2cAMP and papaverine on the TER in confluent MDCK monolayers. Bt2cAMP to a final concentration of 1 mM and papaverine 100 μM were added to both sides of the inserts individually or in combination. All the samples including those as control contained the solvents used to dissolve Bt2cAMP (DMEM/F-12) and papaverine (75% ethanol). The values represent those measured at the end of 2 hour incubation. n = 3.

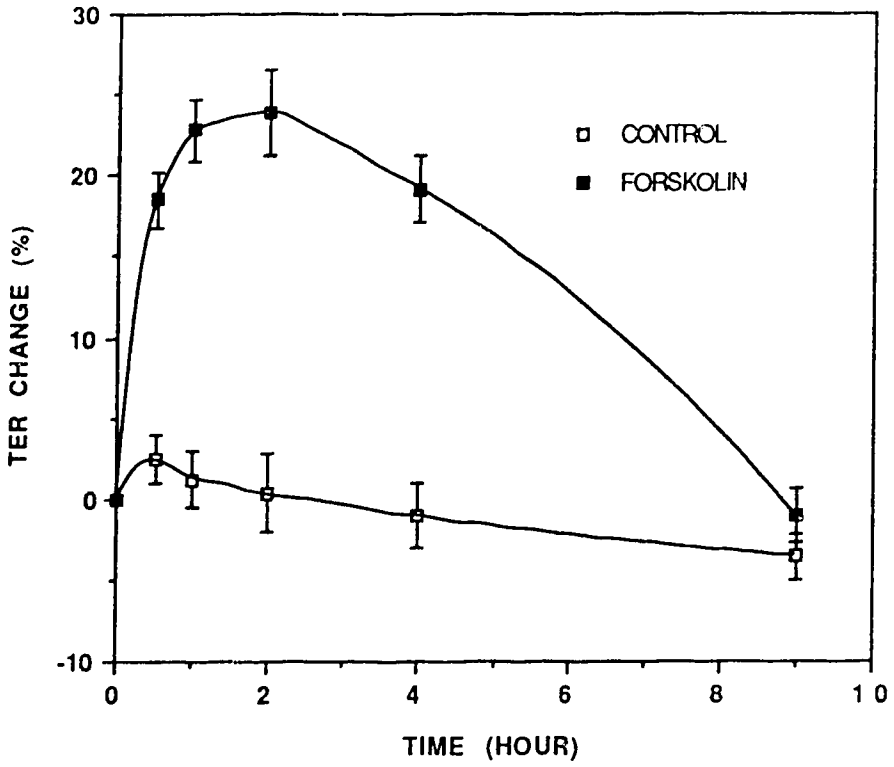

Fig. V-2 Effect of forskolin on TER in confluent MDCK monolayers. Monolayers were prepared as stated in Materials and Methods. Measuring probes and all the solutions to be added to the cells were pre-warmed by putting in the incubator at least 30 minutes before the experiments to minimize the influence by temperature changes. Sterilized solution containing forskolin was added to both sides of the inserts to make a final concentration of 100 μM, and vehicle without forskolin was added to the control. Electrical resistance was continuously recorded up to 9 hours. Longer recording was not attempted, because contamination could be a problem even under the sterile conditions. In this Figure and Fig. V-3, the calculation of TER changes in percentage for each sample was against its own starting value. Each point represents the average from 3 samples.

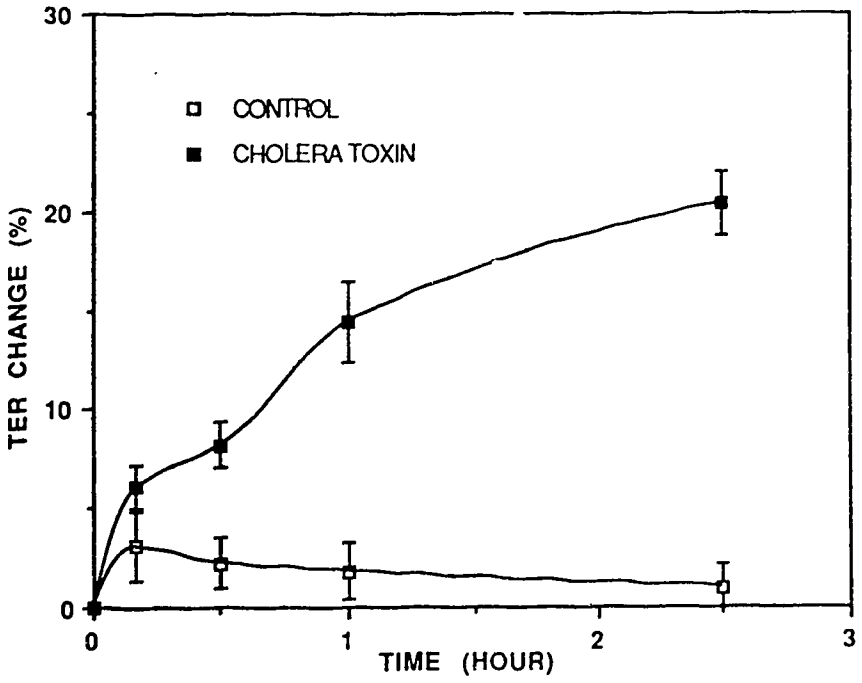

Fig. V-3 Effect of cholera toxin on the TER in MDCK monolayers. Cells were grown for 24 hours to achieve confluence. The experiments were carried out the same way as described in Fig. V-1. Cholera toxin was added at a final concentration of 2 μg/ml. To the control only the corresponding solvent was added. n = 3.

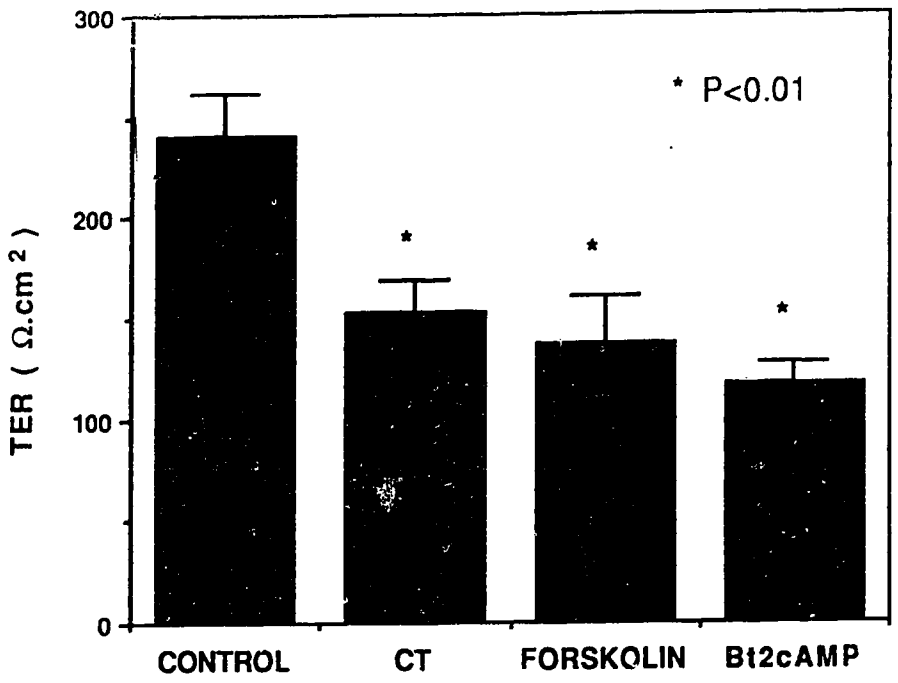

Fig. V-4 Effect of Bt2cAMP, forskolin and cholera toxin (CT) on confluent MDCK monolayer TER after prolonged incubation. Confluent monolayers were prepared by growing the cells for 24 hours. The drugs were added and the cells were incubated for an additional 24 hours. The final concentrations were: Bt2cAMP 1 mM, forskolin 100 μM and cholera toxin 2 μg/ml. The corresponding solvents were added to the control. n = 3.

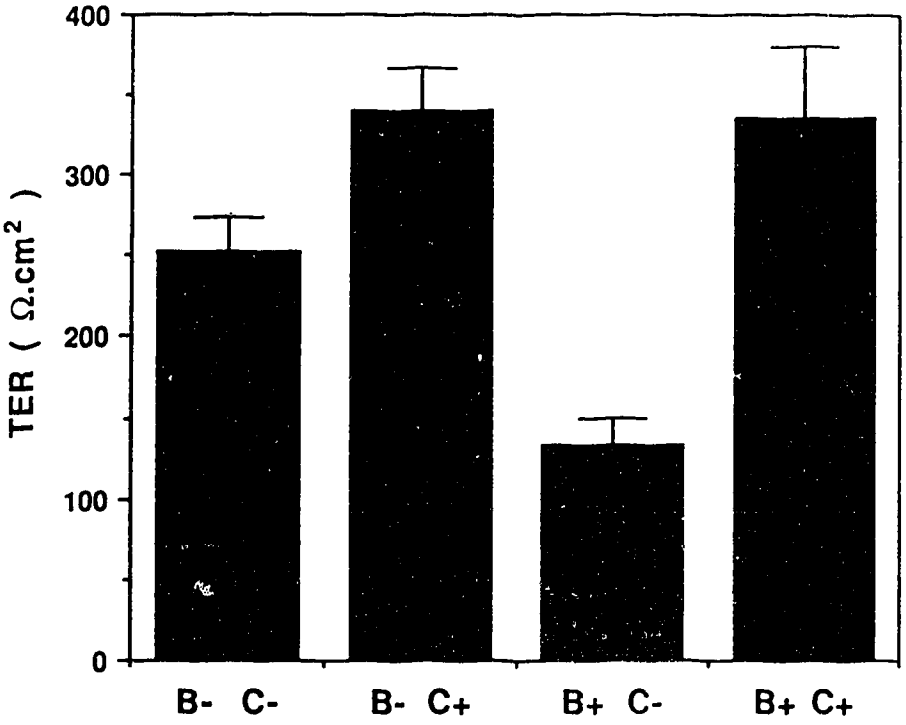

Fig. V-5 Effect of cycloheximide on TER in the confluent monolayers in the presence and absence of Bt2cAMP. Confluent MDCK cells were prepared as described in the Materials and Methods. After 24 growth, cycloheximide (C) at 4 μg/ml and Bt2cAMP (B) 1 mM were added to the indicated samples. - and + represent the absence and presence of cycloheximide or Bt2cAMP, respectively. The monolayers were incubated for an additional 24 hours with the drugs before the TER was measured.

A: ^{3}H-THYMIDINE INCORPORATION (cpm/well)

	control	+ drug
Bt2cAMP	12260 ± 503	15717 ± 281 **
Cholera toxin*	---	14126 ± 488 **
Forskolin	12554 ± 313	14466 ± 236 **

B: CELL PROTEIN (μg/well)

	control	+ drug
Bt2cAMP	512 ± 9.0	578 ± 5.8 **

C: ^{3}H-THYMIDINE UPTAKE (cpm/well)

	control	+ drug
Bt2cAMP	23371 ± 305	26940 ± 639 **

* The same control as for Bt2cAMP was used.

** $P < 0.01$.

Table V-1 Effect of Bt2cAMP, forskolin and cholera toxin on ^{3}H-thymidine incorporation into DNA, its uptake into cells and cell protein. Cells were seeded onto 6-well tissue culture plates (Costar) at a density of $1x10^5$ cells/cm^2. Bt2cAMP, forskolin and cholera toxin at the concentrations of 1 mM, 100 μM and 2 μg/ml, respectively, were added to the indicated samples, and ^{3}H-thymidine at a concentration of 2 μCi/ml was added to all the samples at the time of seeding. Incubation time was 24 hours. At the end of the incubation, cells were thoroughly washed with PBS, and collected into distilled water with a rubber policeman and sonicated. Aliquots of the lysates and TCA precipitates were scintillation-counted to give the incorporation of radioactivity into the whole cells (uptake) and that into DNA. Cell protein was assayed following Lowry et al. (12). The data is expressed in mean ± SE. n = 3.

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CHAPTER VI

EFFECT OF FCCP ON TIGHT JUNCTION PERMEABILITY AND CELLULAR DISTRIBUTION OF ZO-1 PROTEIN IN EPITHELIAL (MDCK) CELLS

KEY WORDS:

Tight junction

FCCP

Madin-Darby canine kidney cell

ZO-1 protein

A version of this chapter has been submitted for publication in Biochim. Biophys. Acta. C. Li, and M.J. Poznansky. It is written in the format of BBA report.

Madin-Darby canine kidney (MDCK) cell represents a cell line of cultured epithelium. Transepithelial electrical resistance (TER) develops in confluent monolayers by virtue of the formation of tight junctions intercellularly (1,2). The tight junction is a dynamic structure which has been shown to be regulated in MDCK and other types of epithelial cells by many factors. For example, cytoskeleton-active drugs (3,4,5,6), cyclic AMP (7), protein kinase C (8), and calcium ion (2) have all been shown to alter TER. The cytoskeleton has been thought to play a key role in the control of the tight junction (4,5). The protein ZO-1 is a well characterized protein which has close association with tight junctions in a variety of epithelial and endothelial cells (9,10). In this study, we examined the effect of the uncoupler of oxidative phosphorylation carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) on the MDCK monolayer TER, an indicator of tight junction dynamics, and on the cellular staining pattern of the protein ZO-1.

For the measurement of TER, MDCK cells were seeded at high density, 1×10^6 cells/cm^2, onto the porous filter inserts (24.5 mm diameter and 0.4 microns pore size; Nucleopore). Cells were allowed to grow for 24 hours in a mixture of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% medium F-12 with 10% bovine serum (FCS) (all from GIBCO) and 1% penicillin-streptomycin (Sigma) under an atmosphere of 5% CO_2 and 95% air. Such monolayer preparations could develop net TER of 350 to 500 ohms.cm^2. A Millicell-ERS apparatus (Millipore) was used to measure the TER. Fig. VI-1 shows the effect of varied concentrations of FCCP on the TER. FCCP was dissolved in a small volume of dimethyl sulfoxide (DMSO) and added to the apical side of the monolayers only. FCCP caused a rapid drop in the TER in

a dose-related manner. Steady state was reached in less than 1 minute.

After the treatment with FCCP, cells in monolayers were examined for viability. Trypan-blue exclusion indicated that there was no viability change after the treatment with 2 μM FCCP for 1 minute, the condition used in both Figs. VI-2 and VI-3. Fig. VI-2 shows the TER recovery following the incubation with FCCP. The TER almost completely recovered in a matter of 1 hour. The time course is similar to that for the TER recovery following calcium ion depletion (1), suggesting a reformation of disrupted tight junctions.

An examination of the distribution of the ZO-1 protein was carried out following the modification of Stevenson *et al.* (9). Confluent MDCK monolayers were prepared on the culture chamber/slides (Nunc), which were coated with 100 μg/ml polylysine (Sigma) in the same way as on the filter inserts. 24 hour old monolayers were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS; GIBCO) for 10 minutes. The permeabilized cells were stained for the protein ZO-1 by the following incubations: (1) anti-ZO-1 monoclonal antibody R40.76 a gift from Dr. B. R. Stevenson in Department of Anatomy and Cell Biology of the University of Alberta) contained in the DMEM with 10% FCS for 1 to 2 hours; (2) a second antibody biotinylated rabbit anti-rat IgG (Vector, CA), 7.5 μg/ml in DMEM plus 10% FCS for 1 to 2 hours; (3) affinity fluorescence label Texas red conjugated streptavidin (Amersham), 4 μl/ml in PBS for 1 hour. Visualization and photography of the protein ZO-1 fluorescence staining were done using a Zeiss ICM 405 fluorescence microscope. Fig. VI-3 shows the FCCP effect on

cellular distribution of the protein ZO-1 by immunofluorescence labelling and on monolayer continuity by phase contrast microscopy in MDCK cells. As shown previously (9,10) ZO-1 protein is exclusively localized to junctional areas or cell peripheries (Fig. VI-3a). Upon FCCP treatment, most of the protein was redistributed from the junctional areas to the cytoplasmic areas (Fig. VI-3b). There were corresponding changes in monolayer continuity by phase contrast microscopy (Fig. VI-3d and VI-3e). Intercellular spaces became wider. After the treated monolayers were incubated in FCCP-free medium for 1.5 hours, they returned to the pretreatment state both in the cellular distribution of ZO-1 (Fig. VI-3c) and in monolayer morphology (Fig. VI-3f).

These data suggest that FCCP can reversably open the tight junctions in MDCK cells. Our initial intention was to examine the effect of energy on tight junction permeability based on the observation in intestinal epithelium by Pappenheimer (11) that transportable nutrients, glucose and amino acids, can increase paracellular or tight junction permeability through increasing intracellular energy levels. FCCP is known as an uncoupler of oxidative phosphorylation (12). It interrupts the coupling between proton gradient and ATP synthesis. We intended to use FCCP here as an energy depleter. To our surprise an examination of intracellular ATP concentration by high performance liquid chromatography (13) showed that 8 μM FCCP did not cause a significant decrease in the ATP concentration in MDCK cells within 4 minutes, during which time FCCP caused significant changes in TER, ZO-1 cellular distribution and cell morphology. In the presence of FCCP, the intracellular ATP concentration might be maintained by compensatory mechanisms, especially by increased

energy production through glycolysis (14) or it might take longer for ATP stores to begin to be depleted.

Another consequence of transmembrane proton gradient disturbance by FCCP is a change in intracellular pH (15). Measurements showed that FCCP at the concentrations affecting tight junction permeability induced intracellular pH drops (Fig. VI-4). It has been shown that FCCP caused the complete disruption of cellular microtubules in BHK21 cells by affecting intracellular pH (16). By the same token, FCCP could profoundly affect cytoskeleton or tight junction assembly. Comparing the time courses for the TER changes (Fig. VI-1) and intracellular pH changes (Fig. VI-4), a close similarity is observed, suggesting that an intracellular pH decrease could be directly responsible for the increase in tight junction permeability. However, the detailed mechanism is unknown.

It has been shown that upon interruption of tight junctions by calcium ion chelation (15,16), ZO-1 protein is redistributed from cell peripheries to cytoplasm. FCCP caused the same change (Fig. VI-3b) which occurred only in less than a minute. Reconcentration of the protein from cytoplasm to cell peripheries was completed in about one hour (Fig. VI-3c). The data suggest that the cellular distribution of ZO-1 protein closely follo he change in tight junction integrity.

In summary, FCCP caused rapid disruption of the tight junctions in MDCK cells probably by affecting intracellular pH. It also induced cellular redistribution of the tight junction protein ZO-1 at the same time.

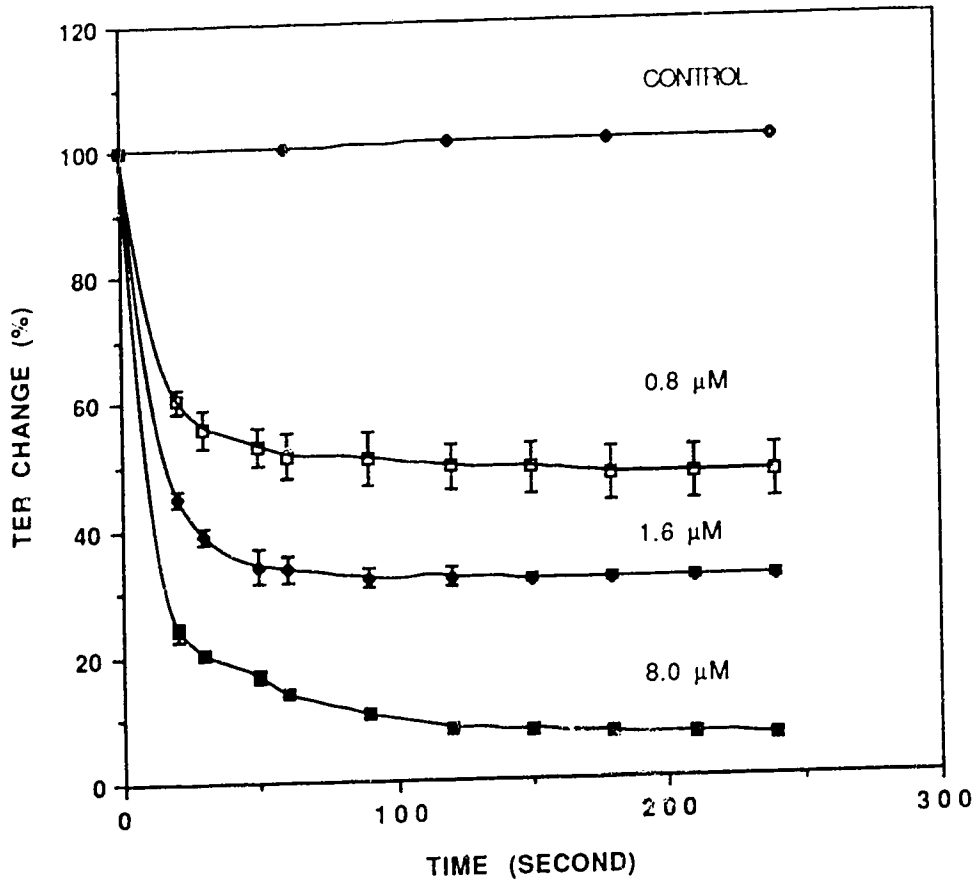


Fig. VI-1 The effect of FCCP on the TER of confluent MDCK monolayers. The concentrations of FCCP in μM used were indicated. Percentage changes in TER for each individual sample was calculated against its own starting TER level before the average of the values from 3 samples for each curve were obtained. The experiments were done in the medium DMEM/F-12 (50%/50%) plus 10% FCS. The vehicle DMSO was added to the control.

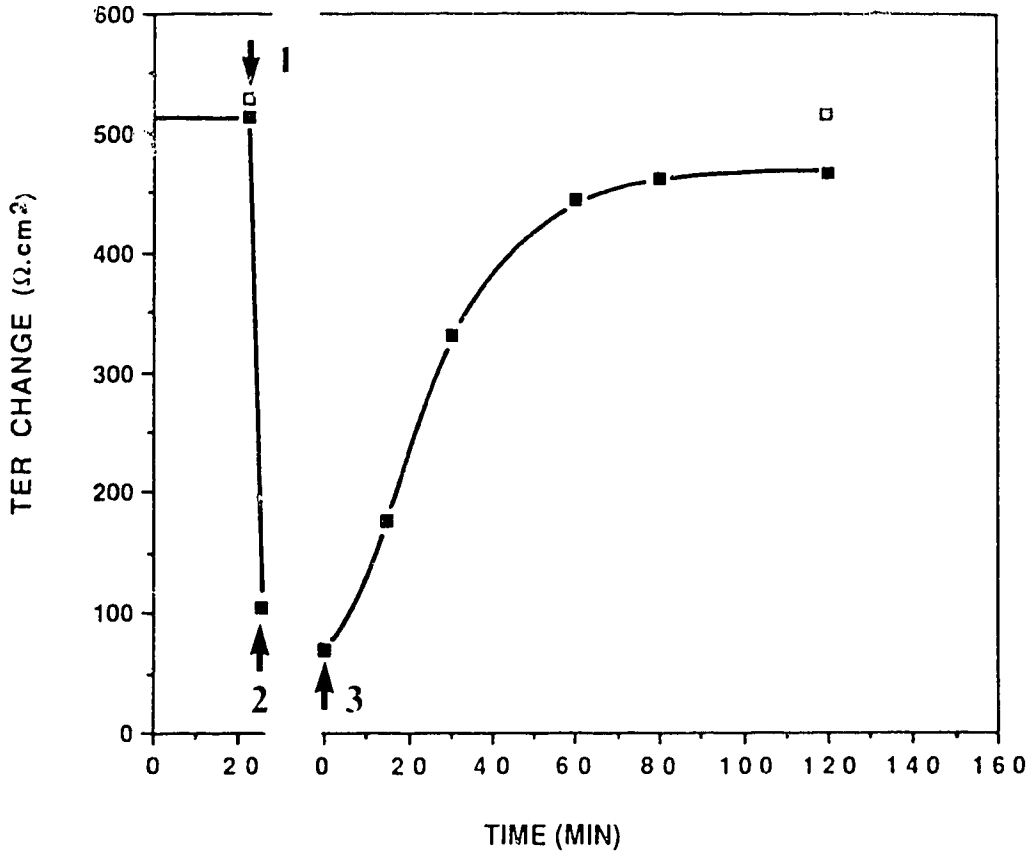
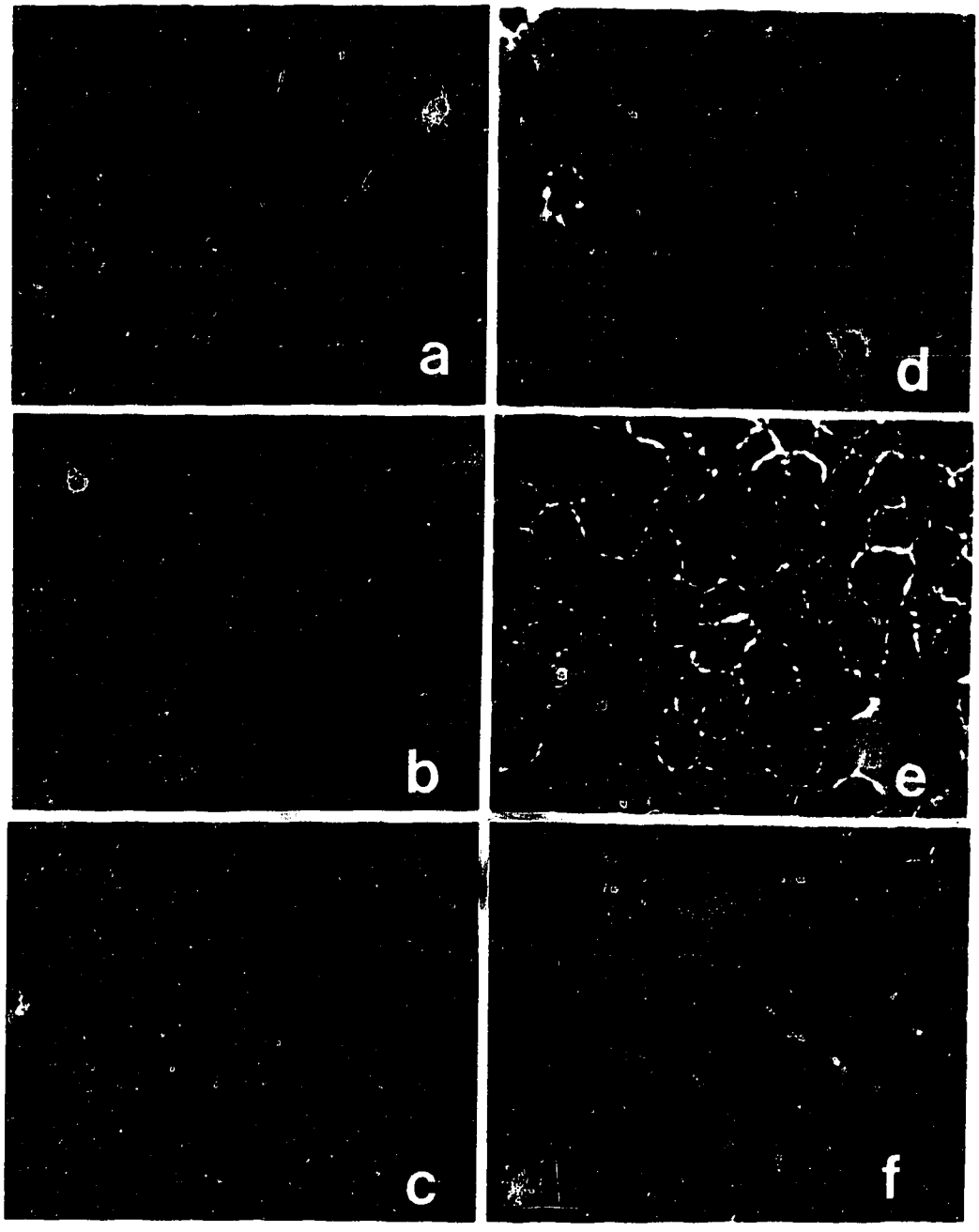


Fig. VI-2 Post-FCCP recovery of MDCK monolayer TER. The cells were first incubated with 2 μM FCCP for 1 minute in DMED/F-12 (50%/50%) with 10% FCS, then washed with PBS twice, and changed to FCCP-free medium (the same medium as the above) to examine the time course of TER recovery. Arrows indicate the addition of FCCP (1), the PBS washes (2) and the change to FCCP-free medium (3). Control samples (open squares) were subject to the same handling as the test samples (solid squares). The standard errors were less than 10% (n = 3).

Fig. VI-3 The effect of FCCP treatment on the distribution of the protein ZO-1 in MDCK monolayers. Immunofluorescence photomicrographs (a, b, c) are shown on the left side and the corresponding phase contrast microscopy of the cell monolayers (d, e, f) is shown on the right side. a and d were not treated with FCCP. b and e were treated with the drug for 1 minute at a concentration of 2 μM. c and f were treated with FCCP the same way as b and e first, then transferred to FCCP-free medium (see Fig. VI-1) and incubated for an additional 1.5 hours. The cells were then fixed for immunofluorescence labelling of the protein ZO-1 and phase contrast microscopy.


a
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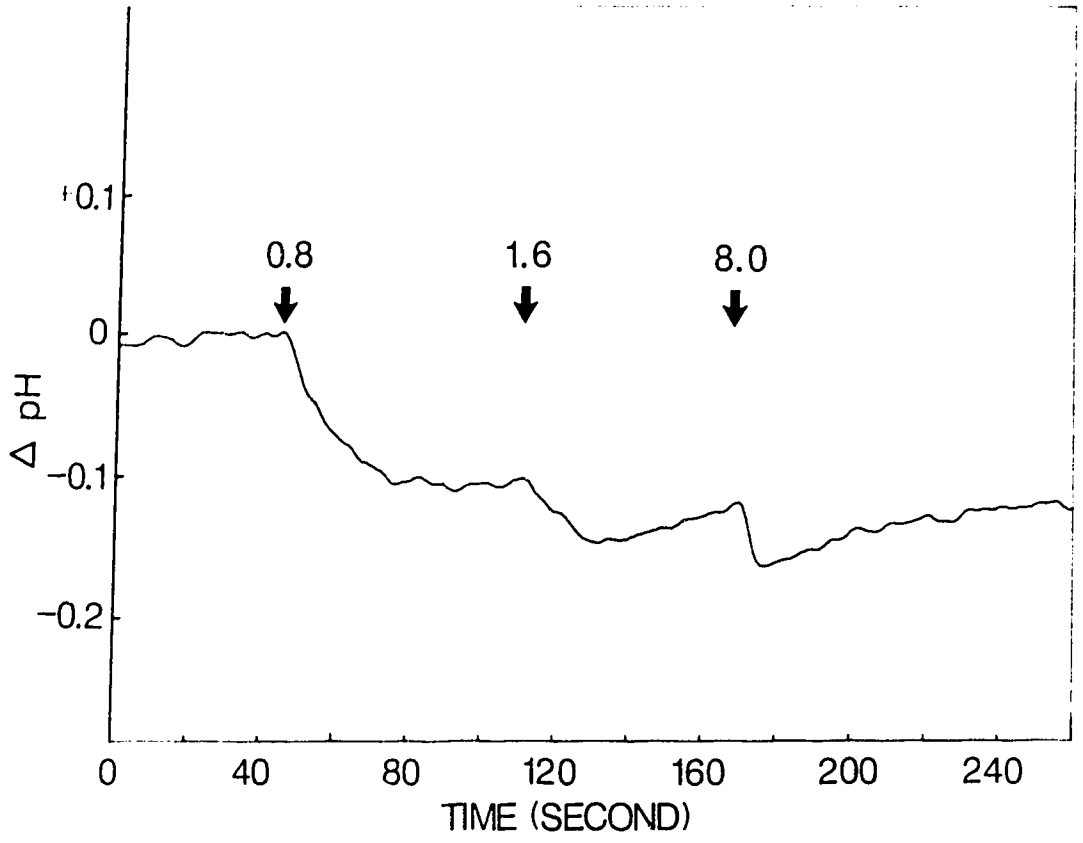

Fig. VI-4 Effect of FCCP on intracellular pH in MDCK cells. Cells in confluent monolayers were trypsinized (0.04% trypsin in calcium-free Puck's saline with EDTA) off the culture flasks, then washed with PBS and incubated with a fluorescent pH indicator, BCECF/AM (Molecular Probes), at 2 μM in PBS for 1 hour at 37°C (19). The cells were then washed 3 times with PBS and resuspended in the buffer at a density of 2 million cells/ml for intracellular pH measurement using a fluorescence spectrophotometer (SLM-8000C, AMINCO) with a thermostatically controlled cell holder fitted with a magnetic stirrer. The numbers indicate the accumulative drug concentrations in μM. The solvent DMSO of the same amount did not cause any change in intracellular pH.

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CHAPTER VII

GENERAL DISCUSSION

In spite of the fact that the thesis covers a broad range of topics, it is focussed on one major theme: the properties of cell barrier or monolayer barrier. Our initial interest was to examine such properties in endothelial cells in order to provide a theoretical basis and practical means for improving the controlled delivery of drugs in circulation (Simionescu et al., 1987, Poznansky and Juliano, 1984). Unfortunately, the endothelial culture we used (bovine aortic endothelium) generates a very low transepithelial electrical resistance (TER) (<30 ohms.cm^2) in monolayer under *in vitro* conditions. In other words, the endothelial monolayer is quite leaky, which makes it difficult to directly measure the transcellular transport across the cell monolayer and to study the regulation of the paracellular or tight junction permeability. For this reason we used epithelial cells in many instances as a substitute for the endothelial cells, since these two types of cells have many properties in common as discussed in Chapter I.

Epithelial monolayers generally develop high TER compared to the endothelial counterpart due to the well-developed tight junctions. As a result, both paracellular transport and transcellular transport can be exmained with ease. We think that both pathways are of physiological significance in the transport of substances across endothelial and epithelial layers.

LDL represents a macromolecule for which there is a receptor on the plasma membrane of cells. We used it as a model molecule to study transcellular transport and other properties of macromolecules because it is well characterized and extensively studied in our laboratory. Poznansky and Julian (1984) hypothesized that macromolecules, because of their size, should get across cells by transcytosis, possibly mediated by their receptors. There is a relatively large number of studies demonstrating transcytosis of macromolecules by using morphological procedures (Simionescu et al., 1987). Generally, there is a lack of investigations of such process by using the procedure as we used (Chapter II), i.e., by quantitatively measuring the amount of a particular substance reaching the other side of a cell monolayer directly.

For most polarized proteins (See Table I-1) asymmetry is anatomical. The asymmetry for LDL receptors seems to be mainly functional. Biochemically the receptor on the apical side of the [illegible] monolayer behaves very much like the basal one as indicated by binding assays and immunoblotting, (Chapter II). However, they have different functions with different localizations. It is easy to understand the properties of the basal LDL receptor, whereas it is not so easy to understand those of the apical receptor. The LDL bound to the basal LDL receptor probably goes to a different intracellular pool from that bound to the apical receptor. One pool is predominantly for degradation and the other for transport across the cell. This is very likely accomplished by the sorting system of the cells, as discussed in Chapter I. However, the detailed mechanism is yet to be defined.

We also examined receptor-mediated transcytosis of LDL in the confluent bovine aortic endothelial monolayers (not shown). The transport of ^{125}I-LDL in the presence and absence of excess amount of cold LDL was not statistically different. We believe that there should be receptor-mediated transcytosis for LDL in endothelium if it occurs in epithelium, since endothelium generally has higher transcytic activity. Failure to detect it may be attributed to the fact that endothelial monolayer is much more leaky than the epithelial counterpart, at least in culture, and paracellular transport so overwhelms the transcellular one so that receptor-mediated transcytosis, if existing, becomes undetectable. As a matter of fact, receptor-mediated transcytosis of LDL has been shown to occur by morphological study (Hashida et al., 1986). Our study with epithelium may provide additonal evidence for this.

The second main area we have worked on is paracellular transport pathway or tight junction permeability in MDCK cells and in bovine aortic endothelial cultures. Biochemical studies of the tight junction protein ZO-1 are made possible by the monoclonal antibody anti-ZO-1 provided by Dr. Bruce R. Stevenson in the Department of Anatomy and Cell Biolgoy, The University of Alberta. We have been able to show the presence of the protein ZO-1 in cultured bovine aortic endothelium, a typical endothelium, and confluence-dependent expression of the protein in the cell type. By extrapolation, it can be safely speculated that the protein is present on most of the other endothelial types.

As described in Chapters IV and V, tight junctions can be regulated by two most universal intracellular second messenger systems: cAMP and

protein kinase C. Even more significant is the fact that the two systems work in the directions opposing one another. It makes sense from a physiological point of view.

The study of the effect of FCCP on tight junciton permeability is based on the observation by Pappenheimer (1987) in intestinal epithelium that the increase in tight junction permeability [illegible] transportable nutrients (glucose, amino acids, etc.) is energy [illegible]d. We expected the uncoupler FCCP to make the tight junctions of the MDCK monolayer tighter. Instead, it caused the tight junctions to become more permeable in a dose-dependent manner.

The effect of FCCP might be due to intracellular pH disturbances or by other mechanisms. However, what is novel is not how FCCP causes this effect but the demonstration that the tight junction is an extremely dynamic structure can be induced to disassemble and reassemble at a very high speed.

Using the techniques of immunofluorescence labelling, the ZO-1 protein was shown to diffuse out of junctional areas when monolayer TER was decreased in response to PMA (Chapter V) and FCCP (Chapter VI). Similar results were reported with calcium depletion (Siliciano and Goodenough, 1988). It is evident that the increase in tight junction permeability is much more than a simple increase in intercellular spaces. There are also dramatic changes occurring intracellularly as demonstrated by the staining of the ZO-1 protein, though we are not sure whether these changes are causes or consequences.

Our results give support to the tight junction model both by Madara (1989) and Stevenson et al. (1988). The ZO-1 protein may be affected directly by the regulators. It is also likely that the protein is affected through cytoskeleton, as suggested in the Madara model. It is easier to explain the regulatory effect of cAMP, protein kinase C, FCCP and calmodulin by linking the action to the cytoskeleton.

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SUMMARY

We have demonstrated that:

1. There are two groups of functionally distinct LDL receptors on MDCK epithelial monolayers. The basal receptor behaves like the classical LDL receptors as described in other cell types. However, the LDL receptor on apical side are not subject to regulation by external LDL, which is in contrast to the basal counterpart. The apical receptor is important in mediating LDL transcytosis. Biochemically, these two groups of receptors seem to be the same.

2. The tight junction-associated protein ZO-1 is present in cultured bovine aortic endothelium, a typical endothelium. There is postive correlation between monolayer confluency and the expression of the protein. The protein was also found in a rat intestinal epithelial line IEC-6 and but not found in mouse myeloma cells.

3. Stimulation of protein kinase C by a phorbol ester decreased TER in MDCK monolayers. The TER change was accompanied by a diffusion of the ZO-1 protein out of junctional areas.

4. cyclic AMP caused TER to increase in MDCK monolayers in short-term. The long-term effect of these agents was the opposite, i.e. to decrease the TER, probably mediated by cyclic AMP-induced cell proliferation.

5. FCCP, probably by decreasing intracellular pH, caused TER in MDCK monolayers to decrease and the ZO-1 protein to diffuse out of

junctional areas in a few seconds. TER recovered within an hour once FCCP was withdrawn from the incubation. In the meanwhile, the ZO-1 protein was reconcentrated back into junctional areas.

6. Our data provide supportive evidences for the tight junction models by madara, and Stevenson and co-workers as described in Chapter 1.

Our major contributions to epithelial/endothelial cell functions are as follows:

PROCEDURES:

1. We originated the idea and created the methodology to specifically regulate LDL receptors on different sides of a epithelial monolayer. Such procedures could be used to study other polarized membrane proteins and other properties of epithelial monolayers.

2. We adapted gelatin for coating the membrane filters and combined the coating and sterilizing processes into one single step by boiling the filters in 0.1% gelatin solution. The procedure is economic. It costs tens to hundreds of times less than using fibronectin, polylysine, and even collagen. The coating efficiency is as good as or better than that using the latter attachment factors, as assayed by TER measurements of the MDCK monolayers growing on the filters coated with different factors (not shown). Gelatin has the advantage over fironectin and polylysine in giving a very homogeneous coat. It also has the advantage over collagen in giving enough thickness of the coat. Since it has low solubility at room temperature or 37°C.

Whatever gets on to the filters at 100°C does not come off easily when the filters are cooled.

3. We used fibroblast-conditioned medium to grow the endothelial culture. The conditoned medium dramatically increases the growth rate and slows down the degeneration rate of the cells.

ORIGINAL FINDINGS:

1. Functional asymmetry of LDL receptors.

2. Transcytosis of LDL in epithelium.

3. Confluence-related expression of the protein ZO-1 in endothelial culture.

4. The effect of protein kinase C on the distribution of the ZO-1 in MDCK monolayers.

5. The short-term and long-term effects of cyclic AMP on the TER of MDCK monolayers.