Intercellular Junctional Proteins as Targets for Protein Kinase C-Mediated Signalling Pathways

Marianne Jennifer Ratcliffe

A thesis submitted for the Degree of Doctor of Philosophy

Eisai London Research Laboratories
University College London

1998
Abstract

Intercellular junctions are dynamic structures responding to a variety of physiological and pathological stimuli. They are particularly well developed in epithelia and endothelia, which function as barriers between different body compartments. This barrier function is dependent on intercellular tight junctions, which prevent unrestricted movement of ions and other solutes between the cells. The integrity of the tight junctions is dependent on a second intercellular junction, the adherens junction, which is the site of cadherin-mediated Ca\(^{2+}\)-dependent cell-cell adhesion. Little is known of the molecular mechanisms by which intercellular junctions are dynamically regulated.

Activation of protein kinase C (PKC) is one route by which modulation of junctional integrity can be achieved. I have investigated whether the proteins which make up intercellular junctions are targets of PKC-mediated signal transduction pathways.

p120, a component of the adherens junction, was found to be a target of a PKC signalling pathway. Activation of PKC by phorbol-12,13-dibutyrate (PDB) led to dephosphorylation of p120 and the closely related p100 protein in epithelial and endothelial cell lines. p100 and p120 are capable of cycling between lesser and higher phosphorylated forms, subject to the action of a serine/threonine phosphatase (or phosphatases) and serine/threonine kinase(s). The pathway by which PKC regulates p100/p120 phosphorylation was investigated, as was the effect of dephosphorylation on the stability and cellular distribution of p100/p120.

Tyrosine phosphorylation of junctional proteins has been correlated with disruption of adhesion. A number of cellular proteins were found to become phosphorylated on tyrosine in response to PDB. The tyrosine phosphorylation of these proteins correlates with increased permeability across epithelial cell monolayers; inhibition of phosphorylation using a tyrosine kinase inhibitor blocks the ability of PKC to increase paracellular permeability.

These data demonstrate the presence of novel PKC-mediated signal transduction pathways that may be involved in the regulation of intercellular adhesion.
Acknowledgements

I would like to express my sincere gratitude to Jim Staddon, my supervisor, for his expert guidance, as well as his unfailing patience and good humour. Also to everyone at Eisai for creating a friendly and stimulating environment in which to work. Finally, I would like to thank my parents for their continued support and encouragement.
Table of Contents

TITLE..........................................................................................................................1

ABSTRACT..................................................................................................................2

ACKNOWLEDGEMENTS.............................................................................................3

TABLE OF CONTENTS..............................................................................................4

TABLE OF FIGURES....................................................................................................9

ABBREVIATIONS..........................................................................................................11

1. INTRODUCTION....................................................................................................12

1.1 Physiological importance of intercellular junctions..............................12

1.2 Ultrastructural studies..................................................................................14

1.2.1 The junctional complex.................................................................14

1.2.2 The barrier function of tight junctions..........................................15

1.2.3 The fence function of tight junctions..........................................17

1.3 Molecular components of intercellular junctions................................19

1.3.1 Components of the tight junction..................................................19

1.3.2 Components of the adherens junction......................................22

1.3.2.1 Cadherins.............................................................................22

1.3.2.2 Catenins...............................................................................28

1.3.2.3 Other components of the adherens junction..........................30

1.3.3 Components of the desmosome......................................................32

1.3.4 Other intercellular adhesion molecules........................................34
1.4 Junctions and signalling

1.4.1 Biological regulators of junctions

1.4.2 Second messenger systems

1.4.3 Junctions and the cytoskeleton

1.4.4 Adherens junctions and signalling pathways

1.4.4.1 Phosphorylation and the adherens junction

1.4.4.2 Adherens junctions as regulators of other junctions

1.4.4.3 Neurite outgrowth

1.4.4.4 β-catenin in Wnt/Wg signalling

1.4.4.5 Cadherin as tumour suppressors

1.4.5 Tight junctions and signalling pathways

1.4.5.1 Phosphorylation and the tight junction

1.4.5.2 Homology of tight junction components to signalling proteins

1.5 Aims of thesis

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Equipment

2.1.2 Antibodies

2.1.3 Cells

2.1.4 Stock solutions

2.2 Methods

2.2.1 Resistance and paracellular flux measurements

2.2.2 Treatment with reagents

2.2.3 Cell Lysis and Immunoprecipitation

2.2.4 Electrophoresis and Immunoblotting

2.2.5 Phosphate Labelling

2.2.6 Phosphoamino acid (PAA) analysis
2.2.7 Phosphopeptide mapping .......................................................... 71
2.2.8 [35S]methionine labelling of p120 associated proteins ............ 73
2.2.9 Protein stability determination ................................................. 73
2.2.10 In vitro kinase assays ............................................................. 73
2.2.11 Immunofluorescence .............................................................. 74
2.2.12 Peptide-directed antibody generation ....................................... 75

3. PHOSPHORYLATION STATE OF CADHERINS,
   CATENINS AND p120αα ISOFORMS IN RESPONSE TO
   ACTIVATION OF PKC IN EPITHELIAL CELLS ......................... 77

3.1 Introduction .................................................................................. 77
3.2 Characterisation of the effects of phorbol ester on tight junctions in
   MDCK I cells .................................................................................. 77
3.3 Phosphorylation of the cadherin/catenin complex ......................... 79
3.4 Dephosphorylation of p100 and p120 ............................................ 81
3.5 Phosphate content of E-cadherin, β-catenin and α-catenin ............ 84
3.6 p100/p120 dephosphorylation visualised in western blots ............ 87
3.7 PKC-dependence and reversibility of p100/p120 dephosphorylation ... 87
3.8 Effect of staurosporine and phosphatase inhibitors on p100 and p120... 90
3.9 p100/p120 dephosphorylation in various epithelia ......................... 92
3.10 Discussion ................................................................................. 92

4. MECHANISM AND NATURE OF p100/p120
   DEPHOSPHORYLATION .............................................................. 97

4.1 Introduction .................................................................................. 97
4.2 Phosphopeptide mapping of p100 ............................................... 98
4.3 Dephosphorylation of a p100/p120 breakdown product ................. 101
4.4 Selected kinase inhibitors promote dephosphorylation of p100 and p120... 104
4.5 Identification of potential GSK-3β phosphorylation sites in p100/p120 ... 107
4.6 GSK-3β protein expression and activity in MDCK I cells.................................108
4.7 p100/p120 is not a substrate for GSK-3 β in vitro.............................................112
4.8 Inhibition of GSK-3 β with kinase inhibitors.....................................................112
4.9 The MAP kinase pathway is not the pathway by which PKC
    induces p100/p120 dephosphorylation..............................................................114
4.10 BAPTA does not induce p100/p120 dephosphorylation.................................116
4.11 Discussion............................................................................................................119

5. INVESTIGATING THE PHYSIOLOGICAL
   RELEVANCE OF p100/p120 DEPHOSPHORYLATION......120

5.1 Introduction............................................................................................................120
5.2 p100/p120 dephosphorylation in endothelial cells..............................................120
5.3 p100/p120 dephosphorylation in fibroblasts......................................................121
5.4 PDB effects on tight junctions in Caco-2, MDCK II and LLC-PK₁ cells.............121
5.5 Effect of kinase inhibitors on paracellular transport..........................................128
5.6 E-cadherin-associated p100/p120 is dephosphorylated in response to PDB.....128
5.7 Immunofluorescence studies of adherens junctions in response to PDB.........130
5.8 p100/p120 stability in MDCK I cells.................................................................130
5.9 Discussion..............................................................................................................137

6. PKC AND TYROSINE PHOSPHORYLATION.......................140

6.1 Introduction............................................................................................................140
6.2 Phosphorylation of cellular proteins in PDB-treated MDCK I cells.................140
6.3 p170 solubility.....................................................................................................143
6.4 Tyrosine phosphorylation of proteins in response to PDB
    is inhibited by herbimycin ..........................................................143
6.5 Herbimycin A blocks the increase in paracellular permeability
    in response to PDB..................................................146
6.6 p170 protein co-migrates with ZO-2.................................................................146
6.7 Generation of a peptide-directed ZO-2 antibody ................................................149
6.8 The p170 protein that becomes tyrosine phosphorylated in response to PDB is not ZO-2 ..........................................................152
6.9 Herbimycin A has no effect on the PDB-induced p100/p120 dephosphorylation ........................................................................154
6.10 Discussion ...............................................................................................154

7. GENERAL DISCUSSION .............................................................................159

8. REFERENCES .............................................................................................169
Table of figures

<p>| Fig. 1.1. | Schematic representation of classic cadherin structure | 23 |
| Table 1.1. | The Cadherin superfamily | 25 |
| Fig. 1.2. | p120&lt;sup&gt;wat&lt;/sup&gt; isoform generation by alternate splicing | 31 |
| Fig. 1.3. | Schematic representation of the tight junction and the adherens junction | 33 |
| Table 1.2. | Permeability modulators of endothelia and epithelia | 37 |
| Fig. 1.4. | β-catennin signalling in the Wnt pathway | 48 |
| Table 1.3. | Classification of PKC isoforms | 57 |
| Fig. 3.1. | MDCK I cell monolayer permeability is increased in response to activation of PKC | 78 |
| Fig. 3.2. | Phosphate labelling of cadherin/catenin complex, following activation of PKC | 80 |
| Fig. 3.3. | Dephosphorylation of p100/p120 following PDB-treatment | 83 |
| Fig. 3.4. | Phosphate labelling of E-cadherin and catenins | 85 |
| Fig. 3.5. | The migration of p100 and p120 during SDS-PAGE is increased following PDB treatment of MDCK I cells | 87 |
| Fig. 3.6. | p100/p120 dephosphorylation is due to activation of PKC | 88 |
| Fig. 3.7. | Time dependence and reversibility of the PDB effect | 89 |
| Fig. 3.8. | Effect of staurosporine and phosphatase inhibitors on p100/p120 mobility | 91 |
| Fig. 3.9. | PKC activation induces a p100/p120 mobility shift in a number of different epithelial cell lines | 93 |
| Fig. 3.10. | Schematic representation illustrating how PKC activation may lead to dephosphorylation of p120 | 95 |
| Fig. 4.1. | Trypsin phosphopeptide map of p100 from DMSO and PDB-treated MDCK I cells | 100 |
| Fig. 4.2. | Thermolysin phosphopeptide map of p100 from DMSO and PDB-treated MDCK I cells | 103 |
| Fig. 4.3. | A 70 kD fragment of p120 is dephosphorylated in response to PDB | 105 |
| Fig. 4.4. | Effects of kinase inhibitors on p100/p120 | 106 |
| Fig. 4.5. | p120 amino acid sequence (murine) | 109 |
| Fig. 4.6. | GSK-3β in MDCK I cells | 110 |
| Fig. 4.7. | GSK-3 β peptide substrate phosphorylation | 111 |
| Fig. 4.8. | p100 and p120 are not substrates for GSK-3 β in vitro | 113 |
| Fig. 4.9. | GSK-3β inhibition by various kinase inhibitors | 115 |</p>
<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.10</td>
<td>p100/p120 dephosphorylation and the MAP kinase pathway</td>
<td>117</td>
</tr>
<tr>
<td>4.11</td>
<td>BAPTA and p100/p120 electrophoretic mobility</td>
<td>118</td>
</tr>
<tr>
<td>5.1</td>
<td>p100/p120 band shift in endothelial cells</td>
<td>122</td>
</tr>
<tr>
<td>5.2</td>
<td>Dephosphorylation of p100/p120 in EA.hy926 endothelial cells</td>
<td>124</td>
</tr>
<tr>
<td>5.3</td>
<td>Dephosphorylation of p120 in NIH 3T3 fibroblasts</td>
<td>125</td>
</tr>
<tr>
<td>5.4</td>
<td>Resistance and sucrose flux across different epithelia in response to activation of PKC</td>
<td>127</td>
</tr>
<tr>
<td>5.5</td>
<td>Resistance and paracellular flux across MDCK I monolayers in response to the kinase inhibitors KT5926 and Go 6976</td>
<td>129</td>
</tr>
<tr>
<td>5.6</td>
<td>p100 and p120 remain associated with the adherens junction complex following PDB stimulation</td>
<td>132</td>
</tr>
<tr>
<td>5.7</td>
<td>Location of adherens junction proteins in untreated MDCK I cells and MDCK I cells stimulated with PDB</td>
<td>134</td>
</tr>
<tr>
<td>5.8</td>
<td>Stability of p100/p120 is unchanged after dephosphorylation</td>
<td>136</td>
</tr>
<tr>
<td>6.1</td>
<td>Tyrosine phosphorylation of proteins in MDCK I cells in response to PDB</td>
<td>141</td>
</tr>
<tr>
<td>6.2</td>
<td>Tyrosine phosphorylation in response to PDB in a variety of epithelial cell lines</td>
<td>142</td>
</tr>
<tr>
<td>6.3</td>
<td>Solubility of p170</td>
<td>144</td>
</tr>
<tr>
<td>6.4</td>
<td>Herbimycin A blocks tyrosine phosphorylation in response to PDB</td>
<td>145</td>
</tr>
<tr>
<td>6.5</td>
<td>Herbimycin A blocks PDB-induced permeability increase in MDCK I cells</td>
<td>147</td>
</tr>
<tr>
<td>6.6</td>
<td>ZO-2 co-migrates with p170</td>
<td>148</td>
</tr>
<tr>
<td>6.7</td>
<td>Characterisation of pepZO-2 antibody</td>
<td>150</td>
</tr>
<tr>
<td>6.8</td>
<td>Immunofluorescence staining with pepZO-2 antibodies</td>
<td>151</td>
</tr>
<tr>
<td>6.9</td>
<td>Immunoprecipitation of tyrosine phosphorylated proteins from the TDS insoluble fraction of MDCK I cells</td>
<td>153</td>
</tr>
<tr>
<td>6.10</td>
<td>Herbimycin A does not block p100/p120 dephosphorylation in response to PDB</td>
<td>155</td>
</tr>
<tr>
<td>6.11</td>
<td>Three schematic representations showing how p100/p120 dephosphorylation and tyrosine phosphorylation of cellular proteins may be involved in the regulation of paracellular permeability by PKC</td>
<td>157</td>
</tr>
</tbody>
</table>
### Abbreviations used in text

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DiC&lt;sub&gt;8&lt;/sub&gt;</td>
<td>1,2-dioctanoyl-sn-glycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/radixin/moesin</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole Limpet haemocyanin</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane associated guanylate kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAA</td>
<td>Phosphoamino acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDB</td>
<td>Phorbol-12,13-dibutyrate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TDS</td>
<td>Triton/SDS/deoxycholate</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Physiological importance of intercellular junctions

Cells in complex organisms are grouped into tissues, which carry out specialised functions. Formation and maintenance of tissues requires that each cell of an organism recognise and adhere to its correct neighbours. From the very earliest stages in development, regulation of cell to cell adhesion is important in cell sorting, determination of cell fate, and co-ordination of complex morphogenetic manoeuvres such as gastrulation (Takeichi, 1988). Once development is complete, cells must then maintain their correct three-dimensional location, which again depends on intercellular adhesion mechanisms.

The best characterised intercellular junctions are those of epithelia and endothelia, which form barriers between different body compartments. Epithelia can have a variety of functions, including protection, absorption, secretion and maintenance of ionic and chemical gradients. A good example of a multi-functional epithelia is that of the stomach. This consists of groups of specialised cells which secrete acid, mucus or enzymes into the stomach cavity. Intercellular junctions between these epithelial cells help form an impermeable stomach wall, thus protecting the surrounding tissues from auto-digestion. The integrity of epithelial cell-cell junctions can be dynamically regulated in certain circumstances. For example, in the intestine, junctions between the absorptive epithelial cells open up to facilitate absorption of glucose after a meal (Madara and Pappenheimer, 1987; Pappenheimer and Reiss, 1987). In some instances of wound healing, junctions disassemble to allow migration of cells to cover the damaged area (Nusrat et al., 1992; Hudspeth, 1982).

The vascular endothelium controls exchanges between blood plasma and the interstitial fluid, allowing metabolites to pass from the blood to the tissues, whilst preventing passage of large macromolecules and circulating cells. Endothelial junctions vary in form and function. Those in the capillary beds and postcapillary venules tend to
be leaky, allowing efficient exchange of metabolites between blood and tissue fluid. Conversely, junctions are well organised and prominent in large arteries, where the endothelial barrier must be resistant to high hydrostatic pressure (Palade, 1988). Likewise, well-developed junctions are present between the endothelial cells of the brain capillaries which form the blood-brain barrier. This barrier, by preventing unrestricted passage of substances between blood and brain, ensures the concentration of electrolytes and chemicals in the cerebrospinal fluid remains constant, in spite of fluctuations in the chemical composition of blood that occur due to exercise, digestion, hormone release and other normal physiological processes (for review see Rubin, 1992). Such homeostasis is vital for proper neuronal function in the brain. As with epithelia, the intercellular junctions of endothelia can be dynamically regulated. In inflammation, the endothelium becomes activated and leaky, facilitating entry of inflammatory mediators into the site of infection. Neutrophils bind to activated endothelium and then exit the bloodstream by passing between the endothelial cells (Springer, 1994).

The importance of intercellular junctions in the functioning of epithelia and endothelia is illustrated by the consequences of their disruption. Opening of the junctions between cells of the blood-brain barrier can occur following stroke. This allows build-up of water (oedema) in the brain, which can lead to severe cellular damage (Rowland et al., 1992). Enhanced leukocyte traffic across the blood-brain barrier is seen in multiple sclerosis, where an auto-immune reaction leads to demyelination of nerve fibres, leading to a loss of neuronal function (Compston et al., 1991; Kermode et al., 1990). In epithelial cells, loss or mutation of components of intercellular junctions has been implicated in a variety of carcinomas (Birchmeier and Behrens, 1994). The intestinal epithelial barrier is compromised in patients with Crohn’s disease, a chronic inflammatory bowel condition (Gray, 1995). Pemphigus vulgaris and Pemphigus foliacus are auto-immune conditions in which blistering of the skin occurs due to disruption of adhesion between cells. In both these diseases, the auto-antigen is a component of intercellular junctions (Amagai, 1995).
Understanding how intercellular junctions are regulated will provide insight into a number of physiological and pathological processes. Adhesion mechanisms involved in dynamic changes such as those seen in development have similarities with those involved in the maintenance of epithelial and endothelial cell barriers, and discoveries about the nature of one system has led to insights into regulation of the other. The earliest information as to nature of intercellular junctions came from studies in epithelia. Indeed, it was in epithelia that the existence of intercellular junctions was first suspected. Histologists in the late 19th century saw a structure between epithelial cells which was predicted to prevent movement of material between cells. This was named the “terminal bar”. It wasn’t until the latter half of the 20th century, and the advent of the electron microscope, that the detailed nature of this structure could be investigated.

1.2 Ultrastructural studies

1.2.1 The junctional complex

In the early 1960s, a series electron microscope studies, culminating in a landmark paper from Farquhar and Palade, revealed the structure of the terminal bar in epithelial cells (Farquhar and Palade, 1963). A wide variety of polarised epithelia were shown to have an apically located junctional complex, with typically three components. These were described as the tight junction (zonula occludens), the intermediate junction (zonula adherens) and the desmosome (macula adherens). The tight junction was the most apical component, followed by the intermediate junction (now known as the adherens junction) and finally the desmosome.

The tight junction completely circumscribed the cell, like a belt. Contact between adjacent cells was so close at this point that the outer leaflets of membranes from neighbouring cells appeared to fuse. Diffusion of tracer molecules such as ruthenium red or haemoglobin between epithelial cells was blocked at this point of apparent membrane fusion. In those epithelia which develop a high electrical
resistance, movement of ions was shown to be blocked at the tight junction, by visualising the electron dense La$^{3+}$ (Martinez-Palomo and Erlij, 1975).

The adherens junction also circumscribed the cell, and appeared as an electron dense region where membranes of neighbouring cells ran parallel, with an intercellular space of approximately 20 nm. A dense fibrillar mass appeared at the cytoplasmic face, suggesting a link to the cytoskeleton.

The desmosome appeared in the form of circular or oval plaques, forming discontinuous, or button-like attachments, often in parallel, and attached to a dense mass of fibres in the neighbouring cytoplasm.

Electron microscope studies of endothelia showed that these too had a specialised junctional complex (Muir and Peters, 1962). Tight junctions were identified in many types of endothelia. Adherens junctions were also present, but could only be seen using refined fixation techniques (Franke et al., 1988). Desmosomes were not found in these cells; they appeared to be limited to epithelia.

1.2.2 The barrier function of tight junctions

Interest focused on the tight junction, as this formed the functional barrier to passage of material between cells. However, the quality of the barrier varied. Some epithelia showed higher resistance to ions, measured electrically, than others. For example, gall bladder, ileum and renal proximal convoluted tubule epithelia were electrically leaky, and La$^{3+}$ was shown to pass through tight junctions in electron microscope studies (Machen et al., 1972). In contrast, renal distal tubules, stomach, sweat gland or amphibian urinary bladder epithelia possessed high resistance junctions (Friend and Gilula, 1972). Endothelial tight junctions were often leaky to ions, and sometimes even to larger molecules (Karnovsky, 1967). Clearly there was a physiological need for variation in “tightness” of these junctions. Epithelia such as that of the distal tube, sweat gland and stomach need to develop steep ionic and/or pH
gradients to carry out their functions, so have extremely tight intercellular barriers, preventing dissipation of these gradients. In contrast, many endothelia function at sites of transfer of materials from the blood to the tissues; such transport will be more efficient if intercellular junctions are leaky. How was such variability achieved? Freeze-fracture electron microscope studies provided a possible explanation.

Freeze-fracture images showed that tight junctions consisted of a meshwork of parallel strands, with interlinking cross bridges, that extended all around the cell (Goodenough and Revel; 1970, Staehelin, 1973). The strands were visualised as fibres on the P-face (inner leaflet), and complementary grooves on the E-face (outer leaflet). Claude and Goodenough proposed a theory that the number of strands correlated with resistance of the tight junction. This was based on observations from a number of epithelia with varying resistance. Junctions from the electrically leaky proximal convoluted tubule of mouse had very shallow tight junctions, with often just one strand circumscribing the cell. In contrast, electrically tight epithelia, such as the frog urinary bladder, possessed junctions with up to five strands in parallel around the cell. Epithelia of intermediate electrical resistance showed intermediate numbers of these junctional strands (Claude and Goodenough, 1973). Other studies confirmed and extended this hypothesis. Madara and Dharmathaphorn showed that resistance of T84 epithelial cell monolayers increased over several days following plating. This correlated with increasing density of tight junctional strands (Madara and Dharmathaphorn, 1985).

The relationship between resistance and strand number was logarithmic, rather than linear. A doubling of average strand number would correspond to an approximate tenfold increase in resistance. This could not be explained if strands were treated as simple resistors in series (in this case, doubling strand number would double resistance). To explain these results, it was proposed that junctional strands might have pores, which opened and closed randomly. If a particular junction had just one strand, with a randomly opening pore, every time the pore opened, current would pass. In contrast, in a junction with two strands, both pores would have to be open at once to
allow current to pass. This event has a much lower probability. For example, if a pore has a probability of being open of 0.2, junctions containing one strand would be open for 20% of the time. However, in a junction with two strands, the probability of both strands being open at the same time would be 0.2 x 0.2, giving 0.04. So this junction would only be open for just 4% of the time. A mathematical model, proposed by Claude, of how such pores would affect resistance predicted a logarithmic relationship between resistance and strand number, in line with the experimental data (Claude, 1978). The observation that tight junctions showed ion selectivity, with cations selected over anions, and K⁺ and Na⁺ more permeable than Li⁺, also suggested the existence of selective pores (Martinez-Palomo et al., 1980; Madara, 1983; Madara et al., 1986). However, there were situations where the strand hypothesis was not valid. Some electrically leaky junctions were found to have high strand complexity (Martinez-Palomo and Erlij, 1975; Stevenson et al., 1988). In conclusion, although tight junctions with a high number of strands tend to form a high resistance barrier, there must be other, as yet unknown, factors that play a role in junctional permeability as well.

1.2.3 The fence function of tight junctions

The tight junction, by forming a barrier to traffic of ions and small molecules between cells, allows maintenance of the different chemical environments on each side of an epithelium. The generation of these different environments occurs due to epithelial cell polarity; the differential arrangements of ion pumps and transporters on the luminal (apical) and abluminal (basolateral) sides of the cell. For example, generation of the acidic environment of the stomach depends on the presence of H⁺ pumps and Cl⁻ channels on the luminal side of the stomach epithelia (Machen and Paradiso, 1987). In the intestine, transporters on the luminal face of the epithelium allows absorption of glucose. This is then actively transported into the bloodstream via ATP-dependent glucose channels located specifically in the abluminal side of the cells.
(Guyton and Hall, 1996). Several studies in the 1970s and 1980s showed that the apical domain of epithelial cells had a different composition of protein and lipid than the basolateral domain, and the boundary to these regions occurs at the tight junction (Pisam and Ripoche, 1976; Roman and Hubbard, 1984). Dissociation of tight junctions led to a redistribution of apical and basolateral proteins, and a loss of membrane polarity. Use of fluorescent lipid probes showed that lipids of the cell membrane outer leaflet were unable pass the tight junction (Dragsten et al., 1981). Thus tight junctions were termed to have a “fence” function, preventing the mixing of apical protein and lipids with those from the basolateral domain (Diamond, 1977).

Endothelia are also polarised to some extent. The blood-brain barrier in particular shows many differences in composition of proteins between the luminal and abluminal side. These allows the blood-brain barrier endothelium to maintain the specific ionic and chemical environment required in the brain (Betz et al., 1980). Particularly important is the presence of P-glycoprotein on the luminal side of the brain capillaries, which protects the brain environment by actively pumping material, such as anti-tumour drugs, out of the brain capillary endothelial cells (Cordon-Cardo et al., 1989; Tatsuta et al, 1992). Even peripheral endothelia are polarised to some extent, perhaps to allow vectoral discharge of secretory products, such as extracellular matrix components and growth factors (Palade, 1988).

The electron microscope studies provided some useful information concerning the structure and function of tight junctions. However, little was known of the nature and function of the adherens junction or the desmosome. Studies on intercellular adhesion had been limited to epithelia and endothelia, whose well-developed junctional complexes were clearly visible in the electron microscope. The nature of cell adhesion in other tissues, or in more dynamic situations such as development was still obscure. Further elucidation of the nature of tight junctions, and of the other intercellular junctions, would depend on characterisation of their molecular components.
1.3 Molecular components of intercellular junctions

1.3.1 Components of the tight junction

In 1986, the first component of the tight junction was identified. Stevenson et al. used a tight junction-enriched fraction of bile canaliculus membranes as an antigen, to which monoclonal antibodies were developed (Stevenson et al., 1986). One antibody recognised a 225 kD protein that bound to the cytoplasmic face of epithelial tight junctions. This protein was present at the tight junctions in a number of epithelia and endothelia, although the apparent molecular weight as visualised by gel electrophoresis varied in different epithelial cell lines (Anderson et al., 1988). The protein was called ZO-1, for zonula occludens-1.

ZO-1 is a phosphoprotein, and two isoforms have been identified, distinguished by the presence or absence of an alternatively-spliced internal 80-amino acid domain (Willott et al., 1992). Although there is no correlation between resistance of a tight junction and its pattern of ZO-1 isoform expression, Balda and Anderson suggest that there might be functional differences between the two isoforms. The shorter isoform (ZO-1α) is expressed in all endothelial cells, and some specialised epithelial cells. Most epithelial cells express only the longer ZO-1α+ isoform. Endothelial cell junctions, and those epithelial junctions expressing the α isoform tend to be more dynamic than junctions in epithelial cells expressing the α+ isoform (Balda and Anderson, 1993).

A number of other peripheral membrane proteins have been localised to the tight junction. ZO-2 is a 160 kD peripheral membrane protein which coimmunoprecipitates with ZO-1 under conditions which maintain protein-protein associations (Gumbiner et al., 1991; Jesiatis and Goodenough, 1994). A smaller, 130 kD phosphoprotein, ZO-3, is also found in ZO-1 immunoprecipitates under these conditions. These three proteins are related, forming part of the MAGUK family of proteins (see below). Also localised to tight junctions are: cingulin, a 140 kD peripheral membrane protein (Citi et al.,
1988); the 7H6 antigen (Zhong et al., 1993), a 155-175 kD protein; symplekin (predicted molecular weight 126 kD) (Keon et al., 1996); and Rab13, a small GTPase (Zahraoui et al., 1994). ZO-2 and cingulin appear to be specific tight junction proteins (Citi et al., 1989; Jesaitis and Goodenough 1994), whereas ZO-1 has been found in non-tight junctional cell-cell contacts, such as intercalated discs between cardiac myocytes. It is also expressed in cells which lack tight junctions, such as astrocytes (Howarth et al., 1992; Itoh et al., 1993). Similarly, symplekin has also been found at locations other than at tight junctions (Keon et al., 1996).

None of these cytoplasmic proteins are capable of forming the structural barrier to transport between cells. Only a membrane spanning protein would have the potential to form such a barrier. A candidate occluding molecule was identified in 1993. A tight junction-enriched fraction from chicken liver, similar to that used by Stevenson et al. in the identification of ZO-1, was used as an antigen for making monoclonal antibodies. The rationale was that the evolutionary distance between chick and mouse would increase the chance of generating an antibody. A 65 kD protein was discovered that localised to the tight junction strands seen in EM freeze-fracture. This protein was named occludin (Furuse et al., 1993). Analysis of the occludin amino acid sequence predicted four hydrophobic α-helical membrane-spanning regions. Topological similarity to the known transmembrane proteins connexin and synaptophysin suggested that occludin is situated with both the N and C-terminals in the cytoplasm, four transmembrane spans, and two short extracellular loops. Consistent with this topological model, a monoclonal antibody against the C-terminal domain labels the cytoplasmic face of the tight junction (Furuse et al., 1993), and an antibody to an epitope in the first of the putative extracellular domain binds to non-permeabilised cells (Van Itallie and Anderson, 1997).

Several lines of evidence indicate an adhesive function for occludin. Over-expression of chick occludin in insect Sf9 cells promotes formation of occludin-rich cytoplasmic lamellar structures, in which membranes appeared to fuse in exactly the same manner as at the tight junction (Furuse et al., 1996). Expression of occludin in
fibroblasts confers adhesive properties (Van Itallie and Anderson, 1997). Synthetic peptides comprising amino acid residues from the second extracellular domain led to decreased expression of occludin and concomitant disruption of epithelial cell barrier function (Wong and Gumbiner, 1997). There is some correlation between occludin expression and permeability of endothelia; occludin expression is much higher in brain endothelial cells, which have electrically tight junctions, than in electrically leaky peripheral endothelial cells (Hirase et al., 1997). Occludin expression appears to be tightly regulated at the transcription level, with mRNA begin detected in epithelial and endothelial cells, but not in fibroblasts (Saitou et al., 1997).

Balda et al. increased the expression of occludin in MDCK cells, by transfection and expression of exogenous occludin. This led to an increase in paracellular resistance, consistent with increased ‘tightness’ of the tight junction. However, there was a concomitant increase in flux of mannitol and 4 kD dextran (Balda et al., 1996a). Similar results were obtained by another group (McCarthy et al., 1996). This raises questions as to the exact function of occludin. How can this protein decrease permeability to ions, whilst at the same time increasing permeability to larger tracers?

The theory that tight junctions consist of a series of diffusion barriers, each with a fluctuating aqueous pore, as proposed by Claude, might explain how this could occur (Claude, 1978). It would be possible for a tracer molecule, such as mannitol, to pass through these diffusion barriers in a stepwise manner, negotiating one barrier whilst the other barriers in the same junction remained closed. In this way, tracer molecules could pass through a tight junction that was electrically sealed at all times. Balda et al. proposed that occludin might be a component of these fluctuating aqueous pores. Increased occludin expression would perhaps increase the number of these pores, or increase the probability of them being open, allowing increased mannitol flux.

In the same study, transfection of a mutant occludin, lacking the C-terminal domain, was still capable of increasing both resistance and flux. However, in these cells, the tight junction belt was disrupted, showing a discontinuous distribution of occludin. The fence function of the tight junctions was also compromised in these
cells, with an apical lipid marker losing its polarised distribution. Whether this fence defect was due to the mis-localisation of occludin, or specifically to the loss of its C-terminus is not known. Clearly, more experiments will be needed to how tight junctions are constructed, and how they function. There may be other components of tight junctions, as yet undiscovered, which may have a vital functional role.

1.3.2 Components of the adherens junction

1.3.2.1 Cadherins

In 1977, Takeichi demonstrated the existence of Ca\textsuperscript{2+}-dependent, and Ca\textsuperscript{2+}-independent forms of intercellular adhesion (Takeichi, 1977). Many instances of cell-cell adhesion could be disrupted by removal of Ca\textsuperscript{2+} from the extracellular environment. This Ca\textsuperscript{2+}-mediated cell-cell adhesion was shown to be dependent on the presence of certain cell surface proteins. These proteins are the cadherins.

Cadherins bind in a Ca\textsuperscript{2+}-dependent manner to similar molecules on neighbouring cells (Volk \textit{et al.}, 1987; Takeichi, 1991). The originally described "classic" cadherins have an extracellular domain with five structural repeats, a transmembrane domain and a highly conserved cytoplasmic domain (fig. 1.1). Three of the first members of the family to be characterised were designated E-cadherin (epithelial), P-cadherin (placental) and N-cadherin (neuronal), according to the tissues from which they were first purified (Takeichi, 1990). L-cells (which lack endogenous cadherin) transfected with cadherins would adhere to each other in a Ca\textsuperscript{2+}-dependent manner (Nagafuchi \textit{et al.}, 1987; Edelman \textit{et al.}, 1987, Hatta \textit{et al.}, 1988). If L-cells transfected with E-, P-, or N-cadherin were mixed together, they would segregate; those cells expressing E-cadherin formed a specific group, as did the P-cadherin and N-cadherin expressing cells (Nose \textit{et al.}, 1988). Thus, cadherins could provide a mechanism for tissue recognition; cells interacting only with those cells expressing the same cadherin.
Classic cadherins such as E-cadherin have five extracellular repeats (EC1-5), each containing 110 amino acids. The membrane proximal motif contains 4 conserved cysteine residues, which mediate dimerisation with an adjacent cadherin, via disulphide linkages. Each extracellular repeat contains amino acids that contribute to Ca$^{2+}$-binding, and this binding of Ca$^{2+}$ is necessary to hold cadherins in the correct three-dimensional structure required for adhesion. The domain nearest the N-terminus contains the HAV (His Ala Val) motif, which is involved in homophilic binding. Cadherins contain a single transmembrane region (TM), and a cytoplasmic domain which is highly conserved in group I classic cadherins.

Fig.1.1 Schematic representation of classic cadherin structure.
In the classic cadherins, the first extracellular domain, closest to the N-terminus is the most conserved. A number of experiments demonstrated that this N-terminal region was necessary for cell-cell interaction. In particular, a conserved tripeptide sequence in the N-terminal domain, HAV (His Ala Val) was shown to be important (Blaschuk et al., 1990; Geiger and Ayalon, 1992). Mutations in the region surrounding the HAV motif of E-cadherin altered binding specificity; an E-cadherin molecule with mutations in this region become able to bind both E- and P-cadherin (Nose et al., 1990). Recently, the crystal structure of this region has been solved, yielding information about the mechanism of cadherin-cadherin interaction (Shapiro et al., 1995; Overduin et al., 1995). The HAV motif forms part of the interaction domain. Ca\(^{2+}\)-binding regions are present in the ectodomain repeats. These regions, although not forming part of the binding site, are vital in maintaining the correct interacting structure of cadherin. A point mutation in just one of the Ca\(^{2+}\)-binding sites disrupts the ability of E-cadherin to form homophilic interactions.

A large number of family members have since been discovered (see table 1.1, and for review see Herrenknecht, 1996; Suzuki, 1996). The classic cadherin structure, as shown in fig. 1.1, is modified in the different family subgroups. Classic cadherins (group II) have the same structure, but show more variation in sequence, and often substitute different amino acids in the HAV motif. For example, in OB-cadherin this becomes a QAV motif. Desmosomal cadherins possess four or five extracellular domains, and have alternatively spliced cytoplasmic domains (Legan et al., 1992). Protocadherins have additional extracellular repeats, and a cytoplasmic domain completely different from that in the classic cadherins. Other cadherin-related proteins differ widely from the classic structure. For example, the Drosophila fat tumour suppressor gene has 34 tandem cadherin repeats in the extracellular domain. The ret oncogene has just one extracellular cadherin repeat, with an intracellular tyrosine kinase domain. Whilst there is huge structural variety, almost all proteins with the cadherin motif can exhibit Ca\(^{2+}\)-dependent homophilic binding. Individual cell types have their own distinctive pattern of cadherin.
Table 1.1. The Cadherin superfamily.
A large number of cadherin-related proteins have been identified, of which a representative sample is shown here. Four subfamilies can be identified, on the basis of sequence homology. A number of proteins that contain cadherin domains, but have no other common features make up a fifth group. This ‘other cadherin-related proteins’ group thus contains some widely divergent proteins. *Taken from Herrenknecht, 1996.*

<table>
<thead>
<tr>
<th>Cadherin group</th>
<th>Structural features</th>
<th>Examples</th>
<th>Predominant Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classic cadherins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>Five extracellular repeats</td>
<td>E-cadherin</td>
<td>Epithelia, early embryo</td>
</tr>
<tr>
<td></td>
<td>HAV motif</td>
<td>L-CAM</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>Conserved (catenin-binding intracellular domain)</td>
<td>P-cadherin</td>
<td>Placenta, epithelium, endothelium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N-cadherin</td>
<td>Neuronal and mesodermal tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R-cadherin</td>
<td>Retina</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cadherin 5 (VE-Cad)</td>
<td>Endothelia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-cadherin (Cad6)</td>
<td>Foetal kidney, brain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M-cadherin</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OB-cadherin</td>
<td>Osteoblastic cells</td>
</tr>
<tr>
<td></td>
<td>Five extracellular cadherin repeats modified HAV motif</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some sequence divergence from classic group I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmosomal cadherins</td>
<td>Four or five extracellular cadherin repeats</td>
<td>Desmoglein 1</td>
<td>Squamous stratified epithelia</td>
</tr>
<tr>
<td></td>
<td>Extracellular anchor domain</td>
<td>Desmoglein 2</td>
<td>All desmosome bearing tissues</td>
</tr>
<tr>
<td></td>
<td>C-terminal splice variants</td>
<td>Desmoglein 3</td>
<td>Squamous stratified epithelia</td>
</tr>
<tr>
<td></td>
<td>(in desmocollins)</td>
<td>Desmocollin 1</td>
<td>Squamous stratified epithelia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desmocollin 2</td>
<td>All desmosome bearing tissues</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Desmocollin 3</td>
<td>Squamous stratified epithelia</td>
</tr>
<tr>
<td>Protocadherins</td>
<td>More than five cadherin repeats</td>
<td>pc-42</td>
<td>Neuronal tissue</td>
</tr>
<tr>
<td></td>
<td>Non-classic Cytoplasmin domain</td>
<td>pc-43</td>
<td>Neuronal tissue</td>
</tr>
<tr>
<td>Other cadherin related proteins</td>
<td>Group comprises proteins with one or more cadherin repeats. No other common features.</td>
<td>Fat (tumour suppressor)</td>
<td>Drosophila imaginal discs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ret (proto-oncogene)</td>
<td>Haematopoietic cells, peripheral nerves</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Li-cadherin</td>
<td>Liver, intestine</td>
</tr>
</tbody>
</table>
expression, which may be important in cell recognition, and segregation of cells into their appropriate tissues.

Cadherin-mediated cell-cell interaction plays a key role in development. The upregulation and/or downregulation of cadherins is associated with alteration of cell-cell attachments that occur in morphogenesis. Compaction of the mouse embryo at the 8-16 cell stage is a vital morphological event, where the hitherto loosely attached blastomeres become strongly adherent. These cells then become polarised and organised into an epithelium (the blastula). E-cadherin becomes localised to regions of cell-cell contact at this stage (Vestweber et al., 1987), and antibodies to E-cadherin prevent embryo compaction (Hyafil et al., 1980; Vestweber and Kemler, 1984). Synthetic peptides containing the HAV motif, which block homophilic cadherin adhesion, also inhibit compaction (Blaschuk et al., 1990).

The epithelial cells of the blastula express E-cadherin until gastrulation, at which point an invagination occurs, and some of the epithelial cells migrate into the hollow core of the blastula, forming a primitive gut. Gastrulation leads to the generation of three germ layers. Cells lining the gut form the endoderm, whilst the epithelial cells which have remained on the outside form the ectoderm. Some cells escape from the migrating epithelium into the space between endoderm and ectoderm, and these cells will form the mesoderm. This process is crucial, and cells from the three germ layers will have distinctly different fates. During gastrulation, the migrating mesoderm cells stop expressing E-cadherin, and express N-cadherin, whilst the ectoderm, and endoderm still expresses E-cadherin (Takeichi, 1988). This implies that loss of E-cadherin by the putative mesodermal cells causes them to dissociate from the ectoderm and allows them to migrate to their specified location.

A similar pattern is seen in neurulation, where a small region of the ectoderm pinches off to form the neural tube. As this region of the ectoderm invaginates, the cells gradually lose E-cadherin, and expressed N-cadherin. As the neural tube closes, some ectodermal cells lose their E-cadherin expression, break away from the ectoderm,
and migrate through the mesoderm. These are the neural crest cells, and will generate most of the peripheral nervous system. Some neural crest cells make contact with the neural tube, and will go on to form the dorsal root ganglion. These cells start to express N-cadherin at precisely the time they connect to the neural tube, which is also expressing this molecule (Takeichi, 1988).

The correlation between cadherin expression and altered cell adhesion continues in other areas of development. Functional studies have demonstrated the importance of cadherins in development. The E-cadherin gene knock-out is embryonic lethal, with mouse embryos unable form a blastula epithelium (Larue et al., 1994). Injection of N-cadherin mRNA into Xenopus eggs can lead to high ectopic expression of N-cadherin in the ectoderm (which does not normally express this molecule). This prevents separation of the N-cadherin-expressing neural tube from the ectoderm during neurulation (Fujimori et al., 1990; Detrick et al., 1990). Injection of N-cadherin antibody into chick embryos distorts development of the neural tube and the migration of neural crest cells, with cells dissociating, and becoming mis-localised, presumably due to inhibition of N-cadherin-mediated cell recognition and adhesion (Bronner-Fraser et al., 1992). Embryos which lack N-cadherin due to gene knock-out show several morphogenetic defects, including disruption of heart development due to dissociation of the myocytes (Radice et al., 1997). The discovery that there is a large and diverse cadherin family suggests that regulation of cell-cell adhesion during development is likely to be extremely subtle and complex.

Cadherins also have a vital role to play in the mature junctions of epithelia and endothelia. Ca\(^{2+}\) depletion or addition of antibodies to cadherins disrupts cell-cell adhesion when applied to intact epithelial cell monolayers (Vestweber and Kemler, 1985; Wheelock et al., 1987). The cells also lose their epithelial morphology, and take on the appearance of fibroblasts. In contrast, transfection of E-cadherin into non-adhesive cell lines, such as L6 fibroblasts, promotes intercellular adhesion, and change in shape, giving a more epithelial morphology (Matsuzaki et al., 1990; McNeill et al., 1990). In mature epithelia, E-cadherin is localised to the adherens junction (Boller et
The formation of tight junctions and desmosomes, as well as the communicating gap junctions in epithelial cells is dependent on function of E-cadherin. MDCK epithelial cells grown in low Ca\(^{2+}\) medium (which prevents cadherin mediated adhesion) do not form any of the other junctions. Addition of Ca\(^{2+}\) is followed by formation of tight junctions (as measured by establishment of resistance). Formation of gap junctions and desmosomes also follows establishment of adherens junctions (Musil et al., 1990; Lewis et al., 1997). Antibodies raised against E-cadherin prevent formation of other intercellular junctions even in the presence of Ca\(^{2+}\) (Gumbiner and Simons, 1987; Gumbiner et al., 1988).

Cadherins are also expressed in endothelial cells (for review see Dejana et al., 1995). Removal of extracellular Ca\(^{2+}\) increases the permeability of peripheral endothelial cells to tracer molecules. Switching to high Ca\(^{2+}\) caused the permeability to revert to original levels (Alexander et al., 1993). As in epithelial cells, an antibody against cadherin prevents the re-establishment of the endothelial permeability barrier on switching back to high Ca\(^{2+}\). Thus, cadherins play an important role in establishing and maintaining the paracellular barrier in endothelia, as well as that in epithelia.

### 1.3.2.2 Catenins

Expression of E-cadherin in non-adhesive cell lines, in addition to promoting intercellular adhesion, induces a change in cell morphology. This implies an interaction with the cytoskeleton. In classic cadherins, the cytoplasmic domain is highly conserved, and it is this region that mediates the interaction with the cytoskeleton. Deletion of the cadherin cytoplasmic domain disrupts the adhesive function of cadherins (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990), despite the fact that the extracellular domain alone is capable of binding another cadherin molecule (Wheelock et al., 1987). Expression of a truncated N-cadherin, lacking the extracellular domain, in the early Xenopus embryo was found to promote abnormal development (Kintner, 1992). This mutant N-cadherin acted in a dominant negative fashion, presumably by
sequestering some limiting cellular protein(s) away from the endogenous cadherin. The search for such proteins identified three polypeptides that co-immunoprecipitated with E-cadherin in cells extracted with non-ionic detergents. They bound, either directly or indirectly, to the cytoplasmic tail of cadherins (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). These proteins were designated α-,β-, and γ-catenin.

α-catenin is a 102 kD protein that shows homology to vinculin (Nagafuchi et al., 1991; Herrenknecht et al., 1991). Vinculin binds to talin (which binds integrins) and α-actinin (which cross-links actin fibres) at the sites of focal contacts. Under appropriate conditions, vinculin can also bind directly to the actin cytoskeleton (for review see Burridge and Chrzanowska-Wodnicka, 1996). Thus, vinculin is a key part of the mechanism by which integrins are linked to the cytoskeleton. Vinculin and α-actinin have also been found at adherens junction (Volk and Geiger, 1984; Knudsen et al., 1995). α-catenin contains regions homologous to the regions of vinculin responsible for self association. This raises the possibility that α-catenin could act as a linker between E-cadherin (via β-catenin) and vinculin, in a role analogous to that of talin at focal contacts. Additionally, there is some evidence that α-catenin, like vinculin, can form a direct link to the actin cytoskeleton (Ozawa et al., 1990). The importance of α-catenin in adhesion is demonstrated by the fact that PC9 lung carcinoma cells, which lack α-catenin, are only loosely adherent, despite the presence of cadherin and the other catenins. Transfection of α-catenin cDNA into these cells promotes adhesion, and formation of a polarised epithelial layer (Watabe et al., 1994). Depletion of maternal α-catenin mRNA causes disruption of intercellular adhesion at the blastula stage of Xenopus embryos (Kofron et al., 1997).

β-catenin links E-cadherin to α-catenin, and is necessary for cadherin function (Oyama et al., 1994; Jou et al., 1995). However, the requirement for β-catenin in cadherin-mediated adhesion can be abolished if α-catenin is fused directly to the E-cadherin extracellular domain (Nagafuchi et al., 1994). γ-catenin was found to be identical to a known protein, plakoglobin (Knudsen and Wheelock, 1992). Plakoglobin and β-catenin compete for E-cadherin binding. Thus, β-catenin/α-
catenin/E-cadherin complexes are distinct from plakoglobin/α-catenin/E-cadherin complexes (Nathke et al., 1994). Plakoglobin also interacts with desmosomal cadherins, unlike β-catenin, which is specific for adherens junctions (Peifer et al., 1992).

β-catenin and plakoglobin belong to the Armadillo family of proteins. Members of this family contain copies of a 42 amino acid repeat motif that was first identified in the Drosophila segment polarity gene product Armadillo. They include nuclear, junctional and signalling proteins (for review see Peifer et al., 1994). The homology of β-catenin and plakoglobin to various signalling molecules suggested that these proteins may function as regulators of cadherin function, perhaps altering interaction of cadherin with the cytoskeleton in response to signalling pathways. Indeed, β-catenin is now known to be a vital component of an important developmental signalling pathway (see below).

1.3.2.3 Other components of the adherens junction

Vinculin, α-actinin, fodrin and ankyrin (Nelson et al., 1990; Knudsen et al., 1995) have all been localised to adherens junctions, and are probably involved in forming a mechanical link between the cadherin/catenin complex and the cytoskeleton (Tsukita et al., 1992). In addition to these structural components, a number of proteins implicated in signal transduction pathways are associated with adherens junctions. Another member of the Armadillo family, p120, binds directly to E-cadherin (Reynolds et al., 1994; Daniel and Reynolds, 1995; Staddon et al., 1995a; Shibamoto et al., 1995; Aghib and McCrea, 1995). p120 contains 11 copies of the 42 amino acid armadillo repeat, and a number of different isoforms have been identified, all the product of a single gene (see Fig. 1.2 and Reynolds et al., 1992; Mo and Reynolds, 1996). Four of these isoforms have been completely characterised in NIH 3T3 fibroblasts. Two proteins of approximately 120 kD (CAS1A and CAS1B) are expressed, the A isoform differing from the B form by the presence of an additional 21 amino acid region close to
Fig. 1.2 p120^as isoform generation by alternate splicing
At least four isoforms of p120 have been identified. These are the product of a single gene. A. p120 gene structure. Two different start codons (ATG1 or ATG2) can be utilised. Alternate splicing of a 5' region (S1) leads to mRNA encoding p120 (CAS1) or p100 (CAS2) isoforms. p120 (CAS1) is synthesised using codon ATG1; for p100, splicing of S2 removes ATG1, and the next start codon, ATG2, is used. In addition, two isoforms of p100 (CAS2A and CAS2B) and p120 (CAS1A and CAS1B) are generated by the alternate splicing of a 63 base pair region (S2). The isoforms of p120 generated by these splicing events are depicted in B. *Taken from Mo and Reynolds, 1996.*
the C-terminus of the protein, due to an alternate splicing event. Two isoforms of 100 kD, CAS2A and CAS2B are also expressed, again differing by the insertion of the 21 amino acid sequence in the CAS2A form. Translation of the 100 kD isoforms is initiated at an ATG codon downstream of the codon used for the 120 kD isoforms; this codon is spliced out of the mRNA encoding the 100 kD isoforms. p120 was originally discovered as a protein whose phosphorylation on tyrosine residues correlated with transformation in cells transfected with pp60\(^{\text{src}}\) (Kanner et al., 1991). Interestingly, the EGF receptor and c-Erb-B2 tyrosine kinases associate directly with the cadherin/catenin complex (Hotchuetzky et al., 1994; Ochiai et al., 1994; Shibata et al., 1996), and the tyrosine kinases c-Yes, c-Src, and c-Lyn also localise to the adherens junction (Tsukita et al., 1991). There is also evidence that protein tyrosine phosphatases can associate with the cadherin/catenin complex. A PTP1B-like phosphatase associates with N-cadherin (Balsamo et al., 1996). LAR (Leukocyte antigen related protein) and hPTP\(x\) have been shown to associate with \(\beta\)-catenin at the adherens junction (Kypta et al., 1996; Fuchs et al., 1996). The presence of these molecules at adherens junctions provides good evidence that adhesion may be subject to regulation by signalling pathways (see below). Figure 1.3 shows a schematic representation of the major components of tight junctions and adherens junctions, and how they are thought to interact.

1.3.3 Components of the desmosome

Desmosomes provide strong adhesion between cells, conferring resistance to physical stresses and strains. They form part of the junctional complex in epithelia, but are also found in the intercellular junctions of non-epithelial tissues, notably in cardiac muscle (for review see Garrod, 1993; Garrod et al., 1996). Desmosomes appear as button-like plaques to which the intermediate (keratin) filament system is attached (Amagai, 1995). The desmocollins and desmogleins are transmembrane glycoproteins that form subfamilies within the cadherin superfamily. These glycoproteins are thought
Ocludin molecules at the tight junction span the membrane, and may form the functional barrier to ions and small molecules. ZO-1 acts as a scaffold, localising other proteins to the tight junction. The tight junctions is linked into the peripheral actin cytoskeleton, possibly via the interaction of ZO-1 with spectrin.

Cadherin molecules form the intercellular link at the adherens junctions, interacting with cadherins on neighbouring cells via the 5th extracellular domain. The function of cadherins depends on the catenins (designated α, β, and γ) which link them to the actin cytoskeleton. Vinculin and α-actinin are also likely to be important in this linkage. Various signalling molecules (tyrosine kinases such as Src and the EGF receptor, protein tyrosine phosphatases PTP1B, LAR and hPTPε, and the Src substrate p120) are also localised at the adherens junction.
to form the basis of cell-cell adhesion at the desmosome. Pemphigus foliaceus and Pemphigus vulgaris are auto-immune conditions, in which the auto-antigen is desmoglein-1 and desmoglein-3 respectively (Stanley et al., 1986; Amagai et al., 1991; Dmochowski et al., 1994). In these diseases cell-cell adhesion is disrupted. Symptoms include severe blistering of skin, and occasionally of other stratified epithelia.

Desmoplakins are cytoplasmic proteins that are believed to provide a link between the desmosomal glycoproteins and the intermediate filaments (Green et al., 1991; Stappenbeck et al., 1992; Stappenbeck et al., 1993). Plakoglobin is also present at desmosomes, and its importance in this junction is demonstrated by plakoglobin gene knock out mice; desmosomes are severely disrupted, and the animals die of heart failure at mid-gestation, almost certainly due to the lack of desmosomes in the intercalated discs (Ruiz et al., 1996). The homology of plakoglobin to various signalling molecules suggest that it may be important in transducing signals at the desmosome. Desmosomes are known to respond to certain signalling pathways, and there is some evidence that engagement of desmogleins and desmocollins can initiate signalling (Garrod et al., 1996).

1.3.4 Other intercellular adhesion molecules

Intercellular adhesion can also be mediated by molecules which do not form part of the classic junctional complex. Platelet/endothelial cell adhesion molecule (PECAM) is a membrane-spanning protein which belongs to the immunoglobulin superfamily (Newman et al., 1990), and can promote homotypic cell adhesion, via interaction with another PECAM molecule, or heterotypic cell adhesion, by binding to glycosaminoglycans (DeLisser et al., 1993). This molecule is found in platelets and leukocytes, but also in endothelial cells where it concentrates at cell-cell contacts distinct from the adherens or tight junctions (Ayalon et al., 1994). It is able to promote
endothelial cell-cell adhesion, and antibodies prevent junctional formation (Albelda et al., 1991).

Integrins, which generally promote cell attachment to the extracellular matrix, have also been found at cell-cell contacts. αβ₁ and αβ₂ have been found at endothelial intercellular contacts, and antibodies against the β₁ chain increases the paracellular flux of horseradish peroxidase between these cells, suggesting a disruption of monolayer integrity (Lampugnani et al., 1991). β₁ integrins have also been seen at regions of cell-cell contact between keratinocytes (Carter et al., 1990; Larjava et al., 1990).

1.4 Junctions and signalling

1.4.1 Biological regulators of junctions

Intercellular junctions are subject to dynamic regulation. In development, junctions are particularly dynamic as cells separate and migrate, then interact with certain specified structures at certain programmed time points. But even those junctions whose primary function is to provide a rigid barrier to passage of material between cells have to be regulated. For example, absorption of glucose across small intestinal epithelium after a meal is facilitated by opening up of tight junctions (Madara and Pappenheimer, 1987; Pappenheimer and Reiss, 1987). Transepithelial and transendothelial migration of neutrophils in inflammation requires dynamic opening and closing of junctions, as does migration of cells during wound healing. Several biological molecules have been shown to increase or decrease the permeability of epithelial and endothelial cell layers (see table 1.2). Some molecules, such as histamine and adenosine, can have opposite effects on permeability, depending on cellular context (Dejana et al., 1995). In the case of histamine, this heterogeneity can be explained by the differential expression of H1 or H2 histamine receptors on target cells (Takeda et al., 1992). These different receptors trigger different signalling pathways.
Table 1.2. Permeability modulators of endothelia and epithelia.

Note some molecules can cause an increase or decrease in paracellular permeability, depending on the cellular context.
Abbreviations; VEGF, vascular endothelial growth factor; SPARC, secreted protein rich in aspartate and cysteine; LPA, lysophosphatidic acid; TNFα, tumour necrosis factor α; EPF, epithelial permeability factor.

References:

2. Watanabe et al., 1992
3. Yonemaru et al., 1992
4. He and Curry, 1993
5. Ehringer et al., 1996
6. Siflinger-Birnboim et al., 1992
7. Connolly et al., 1989
8. Goldblum et al., 1994
9. Goldblum et al., 1993a
10. Goldblum et al., 1993b
11. Royall et al., 1989
12. Maruo et al., 1992
13. Huynh and Dorovini-Zis, 1993
14. Bussolino et al., 1992
15. Schulze et al., 1997
16. Takeda et al., 1992
17. Haselton et al., 1993
18. Lofton et al., 1990
20. Bottaro et al., 1986
21. Fantone et al., 1980
22. Ma and Pedram, 1996; Gillies and Su, 1995
23. Stoker et al., 1987
24. Tamm et al., 1989
25. Madara and Stafford, 1989
26. Marmorstein et al., 1992
27. Conyers et al., 1990
28. Mullin et al., 1992
29. McRoberts and Riley, 1994
30. Woo et al., 1996
<table>
<thead>
<tr>
<th>Endothelia</th>
<th>Epithelia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increase permeability</strong></td>
<td><strong>Decrease permeability</strong></td>
</tr>
<tr>
<td>Histamine (1)</td>
<td>Histamine (16)</td>
</tr>
<tr>
<td>Adenosine (2)</td>
<td>Adenosine (17)</td>
</tr>
<tr>
<td>Atrial natriuretic peptide (3)</td>
<td>Atrial natriuretic peptide (18)</td>
</tr>
<tr>
<td>ATP (4)</td>
<td>Co-cultured astrocytes (19)</td>
</tr>
<tr>
<td>Thrombin (5)</td>
<td>Noradrenaline (20)</td>
</tr>
<tr>
<td>Bradykinin (5)</td>
<td>Serotonin (20)</td>
</tr>
<tr>
<td>H₂O₂ (6)</td>
<td>Prostaglandin E1 (21)</td>
</tr>
<tr>
<td>VEGF (7)</td>
<td>Prostaglandin E2 (22)</td>
</tr>
<tr>
<td>SPARC (8)</td>
<td>Interferon-α2b (23)</td>
</tr>
<tr>
<td>Lipopolysaccharide (9)</td>
<td>Prostacyclin (22)</td>
</tr>
<tr>
<td>TNFα (10)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-1 (11)</td>
<td></td>
</tr>
<tr>
<td>Interleukin-6 (12)</td>
<td></td>
</tr>
<tr>
<td>Interferon-γ (13)</td>
<td></td>
</tr>
<tr>
<td>Scatter factor/HGF (14)</td>
<td></td>
</tr>
<tr>
<td>LPA (15)</td>
<td></td>
</tr>
</tbody>
</table>
In endothelia, various substances produced during inflammation result in generation of large gaps between cells. Histamine and thrombin generally cause a rapid and transient increase in cell permeability. Cytokines and hypoxia induce more sustained increases in permeability (Dejana et al., 1995). Some white blood cells, such as lymphocytes and monocytes can migrate between endothelial cells under normal conditions, whereas neutrophils and eosinophils require chemotactic stimuli, or endothelial activation by inflammatory mediators (Springer, 1994). Neutrophils binding to endothelia can promote opening of tight junctions (Nash et al., 1987). The permeability of endothelial layers can also be influenced by their surroundings. Cells of the blood-brain barrier have remarkably tight junctions compared to those from peripheral endothelial cells. It seems that this is dependent on a factor, or factors, secreted by the neighbouring astrocytes (Risau and Wolburg, 1990).

A particularly dramatic example of adhesion regulation occurs in response to scatter factor (also known as hepatocyte growth factor). This mesenchymally-derived growth factor promotes dissociation and scattering of epithelial and vascular endothelial cells (Stoker et al., 1987; Bussolino et al., 1992). It also has effects on epithelial morphology, such as promoting formation of branching tubules. A variety of functions have been assigned to scatter factor, including facilitation of angiogenesis, promotion of transendothelial T-cell migration, epithelial-mesenchymal interconversion, and regeneration of tissues after injury (Zamegar and Michalopolous, 1995). All of these biological responses require modulation of intercellular adhesions.

In addition to scatter factor, a number of other biological effectors can alter epithelial cell-cell adhesion. Again, effects are cell type specific. TNFα, epithelial permeability factor, and complement components all promote opening of tight junctions in various epithelia (Mullin et al., 1992; Marmorstein et al., 1992; Conyers et al., 1990). Insulin, insulin-like growth factors and interferon-γ increase permeability of mammary epithelial cells (Madara and Stafford, 1989; McRoberts and Riley, 1994). In contrast, the glucocorticoid dexamethasone decreases permeability between mouse
mammary epithelial cells, a response that is blocked by TGFα or TGFβ (Woo et al., 1996).

### 1.4.2 Second messenger systems

A variety of intracellular messengers have been implicated in regulation of intercellular junctions. In some instances, a link between extracellular permeability regulators and second messenger systems has been established.

**G-Proteins**

G-proteins have been implicated in junctional regulation, although the classes which are involved have still to be defined. Thrombin, bradykinin and histamine receptors are all coupled to G-proteins (Lum and Malik, 1994). These pathways can activate PLCβ, leading to phosphoinositide turnover. Pertussis toxin, which inactivates certain G-protein subunits, increases the permeability of bovine pulmonary endothelial cells (Patterson et al., 1995).

**Phospholipase C**

Phospholipase C (PLC) can affect junctions, via interaction with other second messenger systems. Hydrolysis of both PIP and PI by PLC generates diacylglycerol, an activator of protein kinase C. PLC-mediated hydrolysis of PIP₂ generates IP₃, in addition to diacylglycerol. IP₃ increases permeability of pulmonary endothelial cell layers, perhaps via the IP₃-mediated release of Ca²⁺ from intracellular stores (Patton et al., 1991). Thus, PLC could affect intercellular junctions by activating both the PKC and Ca²⁺ second messenger systems (see below).

**Ca²⁺**

Histamine, thrombin and H₂O₂ promote both a transient rise in intracellular Ca²⁺ and a concomitant increase in endothelial permeability (Lum et al., 1989; Ehringer et
Polymorphonuclear neutrophils (PMNs) migrating across human umbilical
vein endothelial cell monolayers cause similar Ca\(^{2+}\) and permeability increases. Both
the increase in permeability and PMN migration is blocked if intracellular Ca\(^{2+}\) is
clamped at a constant low level (Huang et al., 1993). In T84 epithelial cell monolayers,
use of Ca\(^{2+}\) ionophores to raise intracellular Ca\(^{2+}\) leads to an increase in the permeability
of the monolayer (Tai et al., 1996).

### Protein kinase C

Another important intracellular messenger is protein kinase C (PKC), and in
some cases the effects of Ca\(^{2+}\) on paracellular permeability can be attributed to the
activation of Ca\(^{2+}\)-dependent PKC isoforms. The increase in endothelial permeability in
response to thrombin, bradykinin and H\(_2\)O\(_2\) can be blocked, or attenuated by inhibition
of PKC (Johnson et al., 1989; Lynch et al., 1990; Lum and Malik, 1994). Unlike
thrombin and H\(_2\)O\(_2\), the bradykinin-induced increase in permeability does not require
Ca\(^{2+}\) (Ehringer et al., 1996). It is possible that the effect of bradykinin is mediated via
phospholipase D-mediated generation of diacylglycerol, which leads to activation of
PKC (Lum and Malik, 1994). Treatment of many endothelial cells with diacylglycerols
or phorbol esters to activate PKC increases paracellular permeability (Lynch et al.,
1990), although there are some endothelia in which these reagents confer resistance to
permeability modulators (Yamada et al., 1990). Indeed, activation of PKC acts to
promote the increased electrical resistance that develops across bovine brain microvessel
endothelial cells co-cultured with C6 glioma cells (Raub, 1996).

PKC also has diverse effects on cell-cell adhesion in epithelial cells. Activation
of PKC by phorbol ester or diacyl glycerol has been shown to reduce transepithelial
resistance in a number of cell lines, although the effect is again dependent on cell type
(Ojakian, 1981; Ellis et al., 1992; Soler et al., 1993; Stenson et al., 1993). The basis
for the different effects of PKC activation is not known, although the identification of at
least eleven different isoforms of PKC provide a possible explanation for diverse
effects (for review see Kiley et al., 1995; Nishizuka, 1995).
cAMP

cAMP is an intracellular messenger that generally decreases the permeability of tight junctions. Addition of cAMP increases the resistance of *Necturus* gall bladder epithelium, whilst simultaneously increasing the number of tight junction strands (Duffey *et al*., 1981). cAMP also increases the resistance of tight junctions in brain endothelial cells (Rubin *et al*., 1991). Hypoxia decreases cAMP levels (and increases cell permeability) whereas prostaglandin E1 and E2 increase cAMP (and tend to enhance the barrier function of junctions) (Dejana *et al*., 1995). Treatment of cells with cAMP can block the permeability increase caused by ATP, H$_2$O$_2$, neutrophils, and thrombin (He and Curry, 1993; Suttorp *et al*., 1993; Siflinger-Birnboim *et al*., 1993; Patterson *et al*., 1994). Again there are exceptions; instances where cAMP has been associated with increased vascular permeability (Hempel *et al*., 1996). But generally, the cAMP, presumably acting via PKA, prevents disruption of junctions. cGMP can have similar effects to cAMP, blocking the permeability effects of thrombin and H$_2$O$_2$ in certain endothelia (Lofton *et al*., 1990; Suttorp *et al*., 1996).

Interestingly, intracellular messengers such as PKC, inositol phosphates and Ca$^{2+}$, which tend to promote disruption of established tight junctions, also played a role in *de novo* development of tight junctions. Development of resistance in MDCK cells following switch from low to high Ca$^{2+}$ medium (the ‘Ca$^{2+}$ switch’ experiment) is blocked by clamping intracellular Ca$^{2+}$ at constant levels by loading cell with the Ca$^{2+}$ chelator BAPTA (Stuart *et al*., 1994). Activators of PKC induce formation of tight junctions between MDCK cells even in low Ca$^{2+}$ medium; ie in the absence of cadherin-mediated cell-cell adhesion (Balda *et al*., 1993). The establishment of resistance and recruitment of ZO-1 to tight junctions following the Ca$^{2+}$ switch can be prevented by inhibitors of PKC (Nigam *et al*., 1991; Stuart and Nigam, 1995). PLC may also be involved in establishing tight junctions following the Ca$^{2+}$ switch in MDCK cells, since inhibition of this enzyme blocks the formation of tight junctions. This is likely to be due to an activation of PKC and/or Ca$^{2+}$-mediated signal transduction pathways. There is also some evidence that G-proteins negatively regulate formation of tight junctions.
Addition of AlF₃, which activates G-proteins, blocks development of resistance across MDCK epithelial cell monolayers following the Ca²⁺-switch, and pertussis toxin facilitated the development of resistance. In contrast, cAMP, which tend to enhance the barrier function of established junctions is actually an inhibitor of de novo formation of junctions; addition of cAMP prevents development of resistance across MDCK monolayers following the Ca²⁺-switch (Balda et al., 1991). It seems that similar intracellular pathways can be involved in both the de novo formation of tight junctions, and the disruption of established junctions.

In conclusion, there are multiple second messenger pathways affecting the junctional integrity of epithelia and endothelia. These pathways interact and cross talk with each other in ways that have yet to be properly defined. The effects of specific agents will also vary depending on cellular context. Little is known of how these pathways increase or decrease paracellular permeability. Possible targets are the cytoskeleton and/or components of the intercellular junctions.

1.4.3 Junctions and the cytoskeleton

The cytoskeleton provides a framework which is vital for the integrity of intercellular junctions. Loss of interaction between cadherins and the cytoskeleton by deletion of the cadherin cytoplasmic domain abolishes adhesion. Tight junctions are also linked to the cytoskeleton, presumably via the interaction between ZO-1 and spectrin (Madara, 1987; Madara and Pappenheimer, 1987; Itoh et al., 1991). Disruption of perijunctional actin filaments with cytochalasins led to an increase in paracellular permeability, and disassembly of the tight junction in both epithelial and endothelial cells (Madara et al., 1986; Shasby et al., 1982). It is therefore possible that permeability regulators led to opening of junctions indirectly, via their affects on the cytoskeleton. In endothelial cells, LPA, H₂O₂, thrombin, histamine and TNFα all promote rearrangement of the cytoskeleton, with loss of the peripheral actin band and an increase in stress fibres (Schulze et al., 1997; Johnson et al., 1989; Phillips et al.,
This leads to a contraction of cells, generation of intercellular gaps, and an increased permeability to albumin. Stabilisation of F-actin filaments with phallacidin prevents both actin reorganisation, and the increase in transendothelial permeability in response to thrombin (Phillips et al., 1989).

A possible mechanism for cell contraction involves myosin light chain kinase (MLCK). Phosphorylation of myosin light chain (MLC) by MLCK has been implicated in contraction of endothelial cells. Expression of the catalytic domain of MLCK in MDCK epithelial cells increases both MLC phosphorylation, and paracellular permeability (Hecht et al., 1996). Histamine and thrombin both promote an increase in MLC phosphorylation in endothelia. Inhibition of MLCK with the inhibitor ML-9 abolished both MLC phosphorylation and the increase in endothelial permeability in response to these agents (Garcia et al., 1995; Sheldon et al. 1993).

Protein kinase C has also been shown to phosphorylate MLC (Naka et al., 1983). PKC can also phosphorylate vinculin, caldesmon and vimentin, all of which are linked to the actin cytoskeleton (Werth et al., 1983; Stasek et al., 1992). Thus, the permeability increase caused by this kinase could be due to its affects on the cytoskeleton. Vinculin is a component of adherens junctions, and the PKC-mediated phosphorylation of this protein could, in theory, provide a direct mechanism for disruption of junctions.

The cytoskeleton is also regulated by small GTPases. Both Rho and Rac are required for the establishment of epithelial adherens junctions, and recruitment of actin to these junctions (Braga et al., 1997). Inhibition of Rho, by Clostridium difficile toxin B, leads to a reduction in F-actin, and increased permeability in endothelia. The increased permeability in response to this toxin can be blocked if F-actin is stabilised by phalloidin (Hippensteil et al., 1997). Similarly, inactivation of Rho by treatment with C3-transferase disrupts the organisation and integrity of tight junctions in epithelia (Nusrat et al., 1995). Scatter factor leads to cytoskeletal reorganisation prior to scattering of epithelial cells. Dominant negative forms of Ras and Rac block both
reorganisation of the cytoskeleton, and scattering caused by scatter factor (Ridley et al., 1995). A small GTPase, Rab13, is found at tight junctions, raising speculation that this is involved in regulating interaction of the cytoskeleton with the tight junction (Zahraoui et al., 1994). However, given the function of Rab proteins in targeting of membrane vesicles (Novick and Zerial, 1997), Rab13 is perhaps more likely to be involved in the processes of vesicle recognition and fusion by which membrane proteins are targeted to apical and basolateral domains, since these domains are segregated at the tight junction.

It is clear that the integrity of cell-cell junctions is absolutely dependent on the cytoskeleton. An important question is whether the permeability of junctions is controlled physiologically by modulation of the cytoskeleton, or whether junctions are controlled in a more subtle manner. There is some evidence that junctional proteins themselves are targets of signal transduction pathways.

1.4.4. Adherens junctions and signalling pathways

1.4.4.1. Phosphorylation and the adherens junction

Transfection of MDCK epithelial cells with oncogenic src causes a disruption of cell-cell adhesion, and increased invasiveness into a collagen matrix. E-cadherin and β-catenin become phosphorylated on tyrosine residues in these cells (Behrens et al., 1993). Aggregation of rat 3Y1 fibroblasts, which express P-cadherin, is inhibited upon transfection with v-src. P-cadherin and β-catenin become tyrosine phosphorylated in the transformed cells. Both the phosphorylation of cadherin and β-catenin and the disruption of aggregation can be blocked by addition of herbimycin A, an inhibitor of tyrosine kinases (Matsuyoshi et al., 1992). Similar results were seen in chick embryonic fibroblasts, which express N-cadherin (Hamaguchi et al., 1993). Ras-transformed breast epithelial cells also have an altered morphology, and disrupted cell adhesion. β-catenin and p120 become tyrosine phosphorylated in these cells, and this correlates with loss of β-catenin from E-cadherin complexes. Herbimycin A again
decreased the phosphorylation of β-catenin in Ras-transformed cells, and restored normal epithelial morphology (Kinch et al., 1995). Treatment of MDCK cells with tyrosine phosphatase inhibitors pervanadate and phenylarsine oxide increased permeability of monolayers. This correlated with the tyrosine phosphorylation of β-catenin and p120 as well as components of the tight junctions (see below) (Staddon et al., 1995b). p120 was originally identified as a Src substrate whose phosphorylation correlated with a transformed phenotype. It should be noted that in all these cases, tyrosine phosphorylation was induced under rather non-physiological conditions; v-src/ras transfection and tyrosine phosphatase inhibitors cause widespread tyrosine phosphorylation of cellular proteins. Thus it is difficult to be sure that the permeability effects were specifically due to tyrosine phosphorylation of junctional proteins. However, scatter factor has been shown to promote tyrosine signalling of β-catenin, plakoglobin and p120 (Shibamoto et al., 1995). p120 is also phosphorylated on tyrosine in response to EGF, PDGF and CSF-1 (Downing and Reynolds, 1991). The tyrosine kinases c-Yes, c-Src and c-Lyn have been found at adherens junctions (Tsukita et al., 1991). Taken together, these experiments suggest that tyrosine phosphorylation of adherens junction proteins is important in control of intercellular adhesion. The recent evidence showing tyrosine phosphatases, such as hPTPκ, are located at the adherens junctions is interesting as it provides a potential feedback mechanism for regulation of tyrosine phosphorylation.

1.4.4.2 Adherens junctions as regulators of other junctions

In addition to being targets of signalling pathways, there is growing evidence that junctional proteins are actively involved in initiation of signalling pathways. This is particularly true of the adherens junctions. During the formation of epithelial and endothelial junctional complexes, the adherens junctions plays a pivotal role. Homotypic interaction of Ca²⁺-dependent cadherin molecules between adjacent cells is required for normal assembly of tight junctions, since anti-E-cadherin antibodies block
formation of these junctions (Gumbiner and Simons, 1987). Cells which lack cadherins fail to form desmosomes, even though all the components of desmosomes are expressed. Transfection of these cells with E-cadherin and plakoglobin allowed desmosomes to form (Lewis et al., 1997). Hence adherens junction formation must precede formation of other intercellular junctions. A cell permeable diacylglycerol analogue, DiC8, which activates PKC, promotes tight junction assembly in low Ca2+, and overcomes the anti-E-cadherin antibody inhibition of tight junction formation (Stuart et al., 1994). Inhibitors of PKC block formation of tight junctions following the Ca2+-switch (Nigam et al., 1991; Stuart and Nigam, 1995). These results suggests that PKC may act as part of a downstream pathway, initiated by E-cadherin mediated cell-cell adhesion, to promote tight junction assembly. The central role of adherens junctions in formation of the junctional complex as a whole means that this would be an ideal site for signals that modulate adhesion to be integrated. Any signalling pathway that altered adherens junctions function would be likely to affect other junctions as well.

1.4.4.3 Neurite outgrowth

A monolayer of fibroblasts, transfected with N-cadherin, stimulates co-cultured neurons to send out neurites, whereas a control fibroblast layer which does not express N-cadherin has no effect (Doherty et al., 1991). The act of interaction between N-cadherin ectopically expressed in the fibroblasts and the endogenous N-cadherin of the neurons initiates a signalling pathway in the neurons. Ca2+ influx is important, since inhibition of Ca2+ entry, or inhibition of Ca2+/calmodulin-dependent kinase block this neurite outgrowth (Doherty et al., 1991; Williams et al., 1995). This Ca2+ influx is triggered by arachidonic acid, produced by the action of diacylglycerol lipase on diacylglycerol (Williams et al., 1994a). This signalling pathway also involves a tyrosine kinase since erbstatin, a tyrosine kinase inhibitor, blocks neurite outgrowth (Williams et al., 1994b). The realisation that fibroblast growth factor (FGF) promotes neurite outgrowth via an identical second messenger pathway to N-cadherin, suggested
that FGF receptor tyrosine kinase might be the erbstatin-inhibitable kinase involved in N-cadherin signalling. Antibodies against the FGF receptor inhibited neurite outgrowth in response to N-cadherin (Williams et al., 1994c), as did a dominant negative form of this receptor (Saffel et al., 1997). It is possible that N-cadherin and the FGF receptor interact via an extracellular motif that the FGF receptor shares with cadherins. The current model is that the FGF receptor forms a cis interaction with N-cadherin at the surface of neurons, and thus engagement between N-cadherin on the fibroblast monolayer and the co-cultured neurons leads to activation of the tyrosine kinase domain of the FGF receptor (Williams et al., 1994b). This activates a signalling pathway leading to the neurite outgrowth response.

1.4.4.4. β-catenin in Wnt/Wg signalling

A second example of a component of the adherens junctions involved in signalling pathways is β-catenin, which is involved in the Wnt signalling pathway in vertebrates (Moon et al., 1997; Kuhl and Wedlich, 1997). The Drosophila homologue of β-catenin, Armadillo, is involved in the homologous Wingless (Wg) pathway (Orsulic and Peifer, 1996a; Nusse, 1997). Both Wnt and Wg are secreted glycoproteins that bind to receptors on target cells. The Wnt pathway is important in induction of dorsal mesoderm in vertebrate development. Ectopic expression of β-catenin by injection of mRNA into the ventral region of early Xenopus embryos promotes duplication of the dorso-anterior body axis, giving rise to embryos with two heads (Funayama et al., 1995). Wnt also has this effect, and reduction of β-catenin levels inhibits Wnt-induced axis formation, showing β-catenin acts downstream of Wnt. β-catenin is required for formation of dorsal tissues during normal development (Heasman et al., 1994). This function of β-catenin is separate from its role in junctions; mutant β-catenin that is deficient in cell adhesion can still promote axis duplication (Orsulic and Peifer, 1996b). The Wingless pathway in Drosophila is involved in cell patterning; disruption in this pathway, either by loss of the Wg signal, or mutation in
Fig. 1.4 β-catenin signalling in the Wnt pathway.

(A) In the absence of Wnt signal, GSK-3β phosphorylates β-catenin, and this causes degradation of β-catenin via the ubiquitin pathway. APC forms a complex with β-catenin and GSK-3β, and is necessary for this process.

(B) Wnt binding to its receptor (a frizzled family member) promotes inactivation of GSK-3β, by a pathway that has yet to be fully elucidated. β-catenin is no longer targeted for degradation, and accumulates in the cytoplasm. It is also free to bind the LEF/Tcf family of transcription factors in the nucleus, and promote gene transcription. Activated complexes/enzymes are indicated by *. 

---

Frizzled

![Diagram of the Wnt pathway in the absence of Wnt signal](image)

- GSK-3β
- β-catenin
- APC

Ubiquitin degradation pathway

![Diagram of the Wnt pathway in the presence of Wnt signal](image)

- Wnt
- Frizzled
- GSK-3β
- APC

- Dsh?
- β-catenin
- β-catenin
- β-catenin
- β-catenin

- Altered gene expression

Tcf/LEF
armadillo, leads to abnormal segment polarity.

Genetic analysis of the Wg pathway in Drosophila, combined with work on the role of β-catenin in Xenopus development, and biochemical data from cell lines have all combined to give a model of how these homologous pathways work (fig. 1.3). In the absence of Wnt signal in vertebrates, cytoplasmic β-catenin is rapidly degraded, due to phosphorylation (and activation) of 'destruction domain'. This phosphorylation is carried out by Glycogen synthase kinase-3β (GSK-3β), and degradation occurs via the ubiquitin-proteasome pathway (Aberle et al., 1997). The tumour suppressor protein APC plays a role in this event, forming a complex with GSK-3β and β-catenin. APC is itself a substrate for GSK-3β (Rubinfeld et al., 1996). It seems that this complex as a whole promotes the degradation of β-catenin. Binding of Wnt to its receptor activates a signal transduction pathway, that leads to the inactivation of GSK-3β. β-catenin no longer becomes phosphorylated, so is no longer tagged for degradation, and instead accumulates in the cytoplasm (Yost et al., 1996). β-catenin is then available to bind to transcription factors of the LEF-1/Tcf family. These proteins alone are poor activators of transcription, but the complex of LEF-1/Tcf and β-catenin becomes a potent transcriptional activator (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). This complex binds an appropriate target sequence in a gene promoter, and activates transcription of target genes. A number of genes, such as saimois in Xenopus, have been identified as targets of the Wnt pathway (Brannon and Kimelman, 1996).

The homologous wingless pathway in Drosophila works in a similar way, with inactivation of the GSK-3β homologue Zeste/white 3, and stabilisation of Armadillo, although no role for APC has thus far been discovered in the Wingless pathway. The homeotic gene ultrabithorax has been identified as a target gene for the Tcf/Armadillo transcription activation complex (Riese et al., 1997).
Interestingly, the E-cadherin gene contains a Tcf/LEF-1 binding site, suggesting that β-catenin could regulate cadherin-mediated cell adhesion both by interaction at the adherens junctions, and by controlling E-cadherin expression (Huber et al., 1996).

The involvement of APC in targeting β-catenin for degradation has shed some light on the tumour suppressing ability of this protein. APC was originally identified as a tumour supressor gene, that is mutated in familial adenomatous polyposis coli. This inherited defect predisposes carriers to colon cancer. APC is also mutated in a significant number of spontaneous colon cancers (Polakis, 1995). Recent data show that cancer cells lacking functional APC have elevated levels of β-catenin, a phenomenon which can be reversed by addition of wild-type APC (Munemitsu et al., 1995). This elevated β-catenin is presumably due to a defect in its targeting for degradation, a process that seems to require a complex with APC. A number of carcinoma and melanoma cell lines have been found that have wild type APC, but have mutations in the amino terminal domain of β-catenin. This domain contains the putative GSK-3β phosphorylation site. Thus the ‘degradation domain’ is lost, and levels of β-catenin/LEF-1 complexes are increased (Rubinfeld et al., 1997). The correlation between unregulated β-catenin/LEF-1 activity and tumourogenesis suggests that this complex in involved in regulation of cell growth.

Overexpression of plakoglobin is also able to induce axis duplication in Xenopus (Karnovsky and Klymkowsky, 1995). It is not clear whether this is involved in its own signalling pathways, or whether its homology to β-catenin allows it to substitute for β-catenin in this assay. It is clear that these components of the adherens junction have signalling functions. However, these seem to be independent of functions at the junction.

1.4.4.5 Cadherin as tumour supressors

Carcinomas are cancers of epithelia, and invasive carcinomas tend to lose epithelial differentiation, taking on a more fibroblast-like appearance. In cell lines
derived from carcinomas, those which retained their epithelial morphology were
generally non-invasive, and expressed E-cadherin. In contrast, the carcinoma-derived
cell lines that de-differentiated, taking on a more fibroblast-like morphology were
invasive, and had often lost expression of E-cadherin (Birchmeier and Behrens, 1994).
Tumours with aberrant cadherin expression also show an increased tendency to
metastasise. Invasiveness of a de-differentiated breast carcinoma cell line could be
prevented by transfection of E-cadherin cDNA (Frixen et al., 1991; Miyaki et al.,
1995). Transformation of fibroblasts or MDCK cells with oncogenic src disrupts the
homophilic binding of cadherins. These cells also show changes in morphology, and
show greater ability to invade collagen gels (Behrens et al., 1993). The tyrosine
phosphorylation of catenins in this case may be responsible for this loss of cadherin
function. Thus, E-cadherin can be classified as a tumour supressor, since loss or
disruption of E-cadherin mediated cell-cell adhesion correlates with de-differentiation,
invasion and metastasis.

Recent discoveries about the Wnt/Wg signalling pathways have led to
speculation that the role of cadherin as a tumour supressor may be indirect. E-cadherin
could sequester β-catenin, so it becomes unavailable for signalling and transcription
activation. Indeed, overexpression of cadherin antagonises the signalling function of β-
catenin (Fagotto et al., 1996). Loss of E-cadherin might lead to an increase in
cytoplasmic β-catenin, so promotes cell growth. However, this is speculation, and
such an explanation, although intriguing, may not be required to explain the role of E-
cadherin in suppression of invasion and metastasis. It would certainly not be
unreasonable to suppose that disruption the adhesive functions of E-cadherin leads to
increased invasiveness, since this process involves detachment of cells from the
epithelial monolayer, prior to migration.
1.4.5 Tight junctions and signalling pathways

1.4.5.1 Phosphorylation and the tight junction

Components of the tight junctions are also targets for signalling pathways. Tyrosine phosphorylation of ZO-1 and ZO-2 occurs following treatment of epithelial cells with tyrosine phosphate inhibitors vanadate and phenylarsine oxide, which both promote disruption of tight junctions (Staddon et al., 1995). ZO-1 and ZO-2 become transiently phosphorylated on tyrosine residues in A431 cells treated with EGF (Van Itallie et al., 1995). In these cells, the distribution of ZO-1 changes, becoming focused to apical regions of cell contact. In the kidney, formation of a tight junctional structure between glomerular foot processes is induced by perfusion with protamine sulphate. This process involves the tyrosine phosphorylation and recruitment of ZO-1 to these junctions (Kurihara et al., 1995).

The serine/threonine phosphorylation state of tight junction proteins can also be regulated. ZO-1 from MDCK strain II cells has approximately twice as much phosphate as ZO-1 from MDCK strain I cells (Stevenson et al., 1989). MDCK II cells develop a much lower electrical resistance than MDCK Is, but it has yet to be proved that ZO-1 phosphorylation is responsible for this. Tight junction assembly induced by switching MDCK I cells from low Ca\(^{2+}\) to high Ca\(^{2+}\) is associated with increased phosphorylation of occludin. As tight junctions form, the occludin becomes more phosphorylated, and more detergent insoluble (Sakakibara et al., 1997). This suggests that phosphorylation of occludin might be necessary in the process of tight junction assembly.

All these examples demonstrate that tight junction proteins can be targets of signalling pathways. However, links between protein phosphorylation and integrity of tight junctions are still only correlative.
1.4.5.2 Homology of tight junction components to signalling proteins.

ZO-1, ZO-2 and ZO-3 are members of the MAGUK family of proteins (Willott et al., 1993; Anderson et al., 1995; Stevenson et al., 1996). Members of this family include the product of the Drosophila discs-large gene (DlgA), its human homologue hDlg, p55, PSD-95/Sap 90, and Lin-2 from C. elegans. Discs-large (DlgA) is located at the septate junctions of Drosophila. Strong mutations in the dlg gene are lethal, leading to failure to form septate junctions and inability to develop normal cell polarity. Neoplastic growth of epithelial cells occurs in the imaginal discs of larva of dlg mutants (Bryant et al., 1993). PSD 95 (also known as Sap90) is seen at the synaptic junctions in brain, and p55 is a membrane protein from red blood cells. Lin-2 is involved in signalling during vulval differentiation in C. elegans. Members of this family have three motifs in common; SH3 domains, the guanylate kinase domain and PDZ domains.

SH3 domains mediate protein-protein interactions, binding short proline-rich motifs on target proteins. They are present in a large number of proteins involved in signal transduction pathways (for review see Mayer and Eck, 1995). ZO-1 has 11 SH3 domains in its C-terminal domain (Anderson et al., 1995). The function of the guanylate kinase homology domain is unknown. Guanylate kinases catalyse phosphorylation of GMP to form GDP. However, in MAGUK proteins, key catalytic residues are mutated, rendering the guanylate kinase domain functionally inactive (Anderson et al., 1995).

The PDZ domain (an acronym for PSD-95, DlgA, ZO-1) is also known as the DHR (Discs-large homology) or GLGF domain. These are repeats of about 90 amino acids, which contain the conserved motif GLGF (Gly-Leu-Gly-Phe). The crystal structure has been solved for one of these domains from PSD-95, and from hDlg, and shows that these regions bind the absolute C-terminal ends of their target molecules (Doyle et al., 1996; Cabral et al., 1996). The PDZ domains appear to interact with the last four carboxy terminal residues of their targets. The C-terminal residue of the target is generally hydrophobic, such as valine, and the neighbouring four amino acids may
provide specificity, although the exact nature of this interaction is still to be completely resolved (Saras and Heldin, 1996).

PSD-95 binds to the carboxy terminal tails of Shaker type K+ channels and NMDA receptor subunits. This interaction appears to be mediated via the PDZ domains of PSD-95, and leads to clustering of either the K+ channels or the NMDA receptors (Kim et al., 1995; Kornau et al., 1995). p55 is a MAGUK protein from erythrocytes, and binds to the C-terminal region of the transmembrane protein glycophorin C, probably via the PDZ domain of p55. p55 also binds the Band 4.1 protein (Hemming et al., 1995). This protein is a member of the ERM family of tyrosine kinase substrates which are involved in regulating actin-membrane attachments (Tsukita et al., 1997). Band 4.1 protein regulates the affinity of spectrin for actin (Cohen and Foley, 1980). This raises speculation that ZO-1, like p55 a MAGUK protein, may be a docking point for proteins that regulate perijunctional actin. Indeed, a link between ZO-1 and spectrin has already been established (Itoh et al., 1991). In C. elegans, induction of differentiation of precursor epithelial cells to form the vulva involves signalling via a receptor tyrosine kinase, Let-23. Lin-2 (a MAGUK family member) and Lin-7 (not a MAGUK member, but containing a PDZ domain) interact with Let-23 (Hoskins et al., 1996), and are responsible for its correct localisation to the basolateral domain of the vulval precursor cells. Lin-2 or Lin 7 mutants fail to develop a vulva (Kim, 1995).

PDZ domains are present in a number of other proteins that are not members of the MAGUK family, where it is also involved in mediating protein-protein interactions (Saras and Heldin, 1996).

The presence of SH3 and PDZ domains in ZO-1, ZO-2 and ZO-3 suggests that tight junctions can be involved in signalling. If ZO-1, ZO-2 and ZO-3 have functions analogous to other member of the MAGUK family, they could function as adaptor molecules, linking a number of different proteins, and perhaps localising them to specific membrane domains. ZO-1, which has 3 PDZ domains, and 11 SH3 domains, is known to bind spectrin, occludin, a serine/threonine protein kinase as well as ZO-2 (Itoh et al., 1991; Furuse et al., 1994; Balda et al., 1996b; Jesiatis and Goodenough,
1994). ZO-1 has also been shown link α-catenin with actin when it is at non-tight junctional cell cell contacts (Itoh et al., 1997). Mutation of the region of occludin which binds ZO-1 results in a failure of the mutant to localise to the tight junctions, suggesting that ZO-1 may bind and recruit the correct proteins to the tight junction (Furuse et al., 1994).

1.5 Aims of thesis

Although it is clear that intercellular junctions can be dynamically regulated, the mechanism by which this occurs is not understood. I investigated how a well-established permeability regulator, protein kinase C (PKC), might modulate the function of junctions. The PKC family of serine/threonine kinases has for some time been implicated in the regulation of cell-cell adhesion but, although much is known about these kinases, in molecular terms their effects on adhesion are very poorly understood. Activation of PKC isoforms with phorbol esters or diacylglycerols is a rapid event, occurring within a minute of cell stimulation (Castagna et al., 1982). In most cases this is followed by downregulation of PKC, due to increased degradation of the enzyme. The rate of degradation varies in different cell types, and for different members of the PKC family, but generally occurs 1-2 hours after stimulation with phorbol ester (see Young et al., 1987; Olivier and Parker, 1992; Lu et al., 1998).

Tight junctions in endothelia and epithelia can be dynamically regulated by activation of PKC. Commonly, PKC activation by diacylglycerols or phorbol esters disrupts cell-cell adhesion in established cell layers (Ojakian, 1981; Soler et al., 1993; Stenson et al., 1993). The effect is rapid, and thus due to PKC activation, and not the later downregulation of PKC that occurs in response to these agents. In MDCK I, LLC-PK1, and Caco-2 epithelial cells, the tight junctions provide a barrier to ions, as well as larger molecules, and the integrity of tight junctions can thus be measured by assaying resistance. Disruption of tight junctions allows passage of ions between cell,
manifested by a drop in electrical resistance across the monolayer. This is easily measured in cell layers grown on porous filters, allowing access to both the apical and basolateral sides of the cell. Disruption of junctions can also be assayed by flux of tracer molecules such as $^{14}$C-sucrose between cells.

There are instances where activation of PKC does not increase paracellular permeability. For example a subcloned cell line form LLC-PK₁, termed LLC-PK₁, showed increased resistance in response to PKC activation (Ellis et al., 1992). Many endothelial cell junctions are also disrupted by activation of PKC, but again there are exceptions, with some endothelia showing decreased permeability in response to PKC activation (Lynch et al., 1990; Yamada et al., 1990).

So far, eleven different PKC isoforms have been identified (see Table 1.3 and Nishizuka, 1995). Different cell lines express different isoforms, which could account for the diverse responses to PKC activators. PKCα is involved in the disruption of tight junctions in LLC-PK₁ cell lines, since expression of a dominant-negative isoform in these cells attenuated the resistance drop in response to phorbol ester (Rosson et al., 1997). PKCζ has been localised (by immunofluorescence) to cell-cell contacts in MDCK and Caco-2 monolayers (Dodane and Kachar, 1996). However, this isoform is not activated by phorbol esters, so is unlikely to be directly involved in phorbol ester/diacylglycerol-induced permeability changes. MDCK cells, in which the majority of the following experiments were performed, express a variety of PKC isoforms. To date, expression of PKCα, β, δ, ε, γ, ζ (Cardone et al., 1994; Dodane and Kachar, 1996), and PKCη (Chen et al., 1997) in MDCK cells has been demonstrated.

The mechanism by which PKC promotes disruption of junctions is unknown. I investigated whether any components of intercellular junctional are targets for PKC-mediated signal transduction pathways.
Table 1.3 Classification of PKC isoforms
At least eleven different mammalian isoforms of PKC have been identified to date. Members of the classical group are dependent on Ca\(^{2+}\), and are activated by diacylglycerol (DAG) or phorbol esters. The novel PKC forms are independent of Ca\(^{2+}\), but most can be activated by phorbol esters or DAG. Atypical PKC isoforms are Ca\(^{2+}\) independent, and are not activated by DAG or phorbol esters. Adapted from Nishizuka, 1995.
2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Equipment

The chemicals used were of AR grade or above. Chemicals and equipment were obtained from the sources listed below.

*Advanced Protein Products Ltd., West Midlands, UK:*
- Endothelial cell growth supplement

*Alexis Corporation (UK) Ltd., Nottingham, UK:*
- 4α-PDB

*Amersham International Plc, Buckinghamshire, UK:*
- Hybond ECL nitrocellulose, X-ray hyperfilm

*Beckman Instruments Inc., Buckinghamshire, UK:*
- Ready safe™ liquid scintillation cocktail, scintillation vials
- Beckman scintillation counter

*Beckton-Dickinson and Co., NJ, USA:*
- Falcon tissue culture flasks and dishes, plastic syringes
**BioRad, Hertfordshire, UK:**

Acrylamide-bisacrylamide [37.5:1], ammonium persulphate (APS), β-mercaptoethanol, dithiothreitol (DTT), glycine, sodium dodecyl sulphate (SDS), Temed, Tris, Triton-X100, Tween-20

Gel dryer, imaging densitometer GS-670, Trans-Blot cell system for western blotting

**Boehringer Mannheim UK Ltd., East Sussex, UK:**

α,-macroglobulin, HAT media supplement, sodium deoxycholate

**Calbiochem-Novabiochem, Nottingham, UK:**

1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA), bisindolylmaleimide I, bisindolylmaleimide V, calyculin A, cantharadin, cypermethrin, cytochalasin D, epidermal growth factor (EGF), Gö 6976, Gö 7874, H-7, Hepes (ultrol grade), herbimycin A, KN-62, KT5926, ML-7, okadaic acid, PD 98059, Phorbol-12,13-dibutyrate (PDB), staurosporine

**Citiflour Ltd. Kent, UK:**

Citiflour

**Costar UK Ltd. Buckinghamshire, UK:**

24 mm and 6.5 mm diameter polycarbonate Transwell filters (0.4 μm pore size), Spin-X centrifuge filter tubes.

**Eisai Research Laboratories, Tsukuba, Japan:**

Ro 31-8425
1st Link UK, West Midlands, UK.

Plasma derived serum

Genetic Research Instrumentation Ltd, Essex, UK:

Fuji Medical X-ray film (RX)

Globepharm, Esher, UK:

Foetal calf serum

Hoefer Scientific Instruments, Newcastle, UK:

Dual gel electrophoresis unit, combs and spacers, gel plates

Life sciences-Gibco BRL, Paisley, UK:

Dulbecco’s Modified Eagle Medium (DMEM), phosphate-free DMEM, L-glutamine, Ham’s F10, M199, Minimum Essential Medium (MEM), α-MEM, methionine-free MEM, phosphate-free MEM, penicillin/streptomycin, trypsin

Merck Ltd. BDH, Dorset, UK:

Acetone, ethylenediamine tetraacetic acid (EDTA), ethanol, ethanolamine, formic acid, glacial acetic acid, glycerol, HCl, K₂HPO₄, KCl, KH₂PO₄, methanol, MgCl₂, n-butanol, Na₂HPO₄, NaCl, NaF, NaOH pellets, 5-sulphosalicylic acid, ninhydrin, orthophosphoric acid, paraformaldehyde, pyridine, trichloracetic acid

Cellulose plates, coverslips, microscope slides

Millipore, Bedford, MA, USA:

Millicell-ERS resistance system, Immobilon P

Molecular Probes, Eugene, OR, USA:

1,2-Dioctanoyl-sn-glycerol (DiC₂), rhodamine-phalloidin
Keith Johnson and Pelling Ltd, London, UK:
Kodak TMAX 400 photography films

Pharmacia Biotech Ltd., Hertfordshire, UK:
ECH-sepharose, protein A Sepharose
Affinity columns

Pierce and Warriner UK Ltd., Cheshire, UK:
Keyhole limpet haemocyanin (KLH), N-ethyl-N’-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC),
CelluSep dialysis tubing

Scientific Laboratory Supplies Ltd, Nottingham, UK.
23 gauge syringe needles

Sigma-Aldrich Chemical Company, Dorset, UK:
Adenosine triphosphate (ATP), ammonium bicarbonate, ammonium carbonate, bovine serum albumin (BSA), bromophenol blue, dimethylsulphoxide (DMSO), \( \epsilon \)-dinitrophenyl (DNP)-lysine, ethyleneglycol-bis(\( \beta \)-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA), gelatin, glutaraldehyde, hydrogen peroxide, isobutyric acid, L-lysine, leupeptin, phosphoserine, phosphothreonine, phosphotyrosine, phenylmethylsulphonylfluoride (PMSF), putrescine, sodium azide, sodium orthovanadate, sodium selenide, soybean trypsin inhibitor, sterile phosphate buffered saline (with or without \( \text{Ca}^{2+}/\text{Mg}^{2+} \)), thermolysin, transferrin, Trizma-HCl, xylene cyanol FF

TCS Biologicals, Buckinghamshire, UK:
Phospho-glycogen synthase peptide-2 (GSK-3\( \beta \) peptide substrate), [Ala\(^2\)]-GS peptide-2, GSK-3\( \beta \) recombinant enzyme.
Universal Biologicals Ltd., London, UK:

Ribi adjuvant

Whatman Scientific Ltd, Kent, UK:

3MM filter paper, P81 chromatography paper

Worthington Biochemical Corporation, Freehold, NJ, USA:

TPCK-Trypsin

2.1.2 Antibodies

Antibodies recognising pp120, Erk-2, FAK, Ras, E-cadherin, α-catenin, Paxillin, Cdc2, GSK-3β and phosphotyrosine (RC20H) were from Transduction Laboratories, KY, USA.

β-catenin antibody was a gift from Kurt Herrenknecht (Eisai London Research Labs, London, UK).

ZO-1 antibody was from Zymed, distributed by Cambridge BioScience, Cambridge, UK.

Phospho MAPK antibody was from New England Biolabs Ltd., Hertfordshire UK.

ZO-2 antibody was a gift from Lynne Jesaitis and Daniel Goodenough, Harvard Medical School, Boston, MA, USA.

pepZO-2 antibody was generated as in Materials and Methods.

Rabbit anti-mouse secondary antibodies for immunoprecipitation, FITC-conjugated anti-mouse IgG, FITC-conjugated anti-rabbit IgG and goat γ-globulin were from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.

HRP-conjugated anti-mouse and anti-rabbit antibodies were from Amersham International Plc., Buckinghamshire, UK.
2.1.3 Cells

The following cells were cultured at 37°C in humidified air with CO₂ present at the percentage indicated.

Caco-2 (epithelial cells derived from a human colonic tumour): 5 % CO₂, MEM, 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 % non-essential amino-acids, 1 µg/ml insulin).

LLC-PK₁ (epithelial cells derived from porcine kidney): 5 % CO₂, M199, 10 % FCS, 2 mM L-glutamine. 100 U/ml penicillin and 100 µg/ml streptomycin.

MDBK (epithelial cells derived from bovine kidney): 5 % CO₂, MEM, 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

ECV304 (a cell line derived from human umbilical vein endothelial cells): 5 % CO₂, M199, 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Caco-2, LLC-PK₁, MDBK, and ECV304 cells were obtained from the European Collection of Animal Cell Cultures, Salisbury, UK.

EA.hy926 cells: DMEM, 10% FCS, 10 % CO₂, 1:500 HAT (hypoxanthine 50 mM, aminopterin 0.2 mM, thymidine 8 mM), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. EA.hy926 cells were provided by Cora-Jean Edgell, University of North Carolina, Chapel Hill, USA.

Strain I and II MDCK cells (epithelial cells derived from canine kidney): 5 % CO₂, MEM, 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.
streptomycin. These cells were provided by Barry Gumbiner, Memorial Sloan-Kettering Cancer Center, NY, USA.

RBE4 cells (immortalised rat brain endothelial cells (see Durieu-Trautmann et al., 1993): 5% CO₂, α-MEM: Ham's F10 (1:1), 10% FCS, 0.3 mg/ml geneticin, 1 ng/ml bFGF; 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown on dishes coated with rat tail collagen. These cells were from Pierre Couraud, Université Paris VII, Paris, France.

BAEC (bovine aortic endothelial cells): 5% CO₂, DMEM, 10% FCS, 30 μg/ml endothelial cell growth supplement, 90 μg/ml heparin. Cells plated on dishes coated with 1% gelatin. BAEC were a gift from Roy Bicknell, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK.

NIH 3T3 fibroblasts: 10% CO₂, DMEM, 10% FCS. Cells were from Art Alberts, Imperial Cancer Research Fund, London, UK.

HUVEC (Human umbilical vein endothelial cells) and SAEC (small airway epithelial cells) were from Clonetics, Palo Alto, CA, USA, and cultured according to the manufacturers instructions.

PBEC (Primary pig brain endothelial cells) were a gift from Louise Morgan and Joanna Brashaw, and were isolated and grown by modification of a previously published procedure (Rubin et al., 1991). Brain capillary fragments were plated on dishes coated with rat tail collagen and human fibronectin. The cultures were fed every other day with growth medium composed of 50% astrocyte conditioned medium and 50% DMEM containing 10% plasma-derived serum, 125 μg/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. They were incubated in 10% CO₂ at 37°C. When the endothelial cells were 50% confluent, they were trypsinised and plated onto
collagen-coated polycarbonate Transwell filters (6.5 mm diameter) in the same medium. After 2-3 days on filters, the medium was switched to a 1:1 mixture of astrocyte conditioned medium and N2 medium. N2 medium consists of DMEM (low glucose), 10 µg/ml transferrin, 100 µM putrescine, 30 nM sodium selenite, 100 U/ml penicillin and 100 µg/ml streptomycin. Astrocyte conditioned medium was obtained by culturing astrocytes from the cortex of 0-2 day old rats in DMEM, 10% plasma-derived serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and harvesting the medium every two days.

2.1.4 Stock solutions

Solutions were made up in MilliQ deionised water, unless otherwise indicated.

<table>
<thead>
<tr>
<th>Triton lysis buffer</th>
<th>25 mM Hepes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>2 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.1 M NaCl</td>
</tr>
<tr>
<td></td>
<td>25 mM NaF</td>
</tr>
<tr>
<td></td>
<td>1 mM vanadate (add just before use)</td>
</tr>
<tr>
<td></td>
<td>1 mM PMSF</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml soybean trypsin inhibitor</td>
</tr>
<tr>
<td></td>
<td>0.1 Units/ml α₂-macroglobulin</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml leupeptin</td>
</tr>
<tr>
<td></td>
<td>NaOH until pH 7.4 at 4°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TDS lysis buffer</th>
<th>To Triton lysis buffer add;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5% w/v sodium deoxycholate</td>
</tr>
<tr>
<td></td>
<td>0.2% SDS</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Composition</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SDS lysis buffer</td>
<td>1% SDS&lt;br&gt;25 mM Hepes&lt;br&gt;25 mM NaF&lt;br&gt;2 mM EDTA&lt;br&gt;1 mM vanadate (add just before use)&lt;br&gt;NaOH until pH 7.4 at room temperature</td>
</tr>
<tr>
<td>Triton dilution buffer</td>
<td>2.9% Triton X-100&lt;br&gt;25 mM Hepes&lt;br&gt;0.1 M NaCl&lt;br&gt;25 mM NaF&lt;br&gt;2 mM EDTA&lt;br&gt;1 mM vanadate (add just before use)&lt;br&gt;NaOH until pH 7.4 at 4°C</td>
</tr>
<tr>
<td>Laemmli sample buffer</td>
<td>62.5 mM Tris-HCl pH 6.8&lt;br&gt;2% SDS&lt;br&gt;10% glycerol&lt;br&gt;5% β-mercaptoethanol&lt;br&gt;0.025% w/v bromophenol blue</td>
</tr>
<tr>
<td>Gel running buffer</td>
<td>192 mM glycine&lt;br&gt;25 mM Tris&lt;br&gt;0.1% w/v SDS</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>2% w/v Ponceau S powder&lt;br&gt;30% v/v Trichloroacetic acid&lt;br&gt;30% sulphosalicylic acid</td>
</tr>
</tbody>
</table>
| **Transfer buffer** | 48 mM Tris  
|                     | 39 mM glycine  
|                     | 0.03% w/v SDS  
|                     | 20% v/v methanol  
| **Phosphate buffered saline (PBS)** | 137 mM NaCl  
|                     | 2.7 mM KCl  
|                     | 1.47 mM KH₂PO₄  
|                     | 8.1 mM Na₂HPO₄  
| **Immunofluorescence “block”** | 0.1 M lysine  
|                     | 10% v/v Foetal calf serum  
|                     | 0.01M sodium azide  
|                     | make in PBS (without Ca²⁺/Mg²⁺)  
| **Kinase assay (KA) buffer** | 50 mM Tris-HCl pH 7.4  
|                     | 10 mM MgCl₂  
|                     | 1 μM DTT  
| **PAA buffer, pH 1.9** | 50 ml formic acid (88%)  
|                     | 156 ml glacial acetic acid  
|                     | 1794 ml deionised water  
| **PAA buffer, pH 3.5** | 100 ml glacial acetic acid  
|                     | 10 ml pyridine  
|                     | 1890 ml deionised water  

KPi buffer (for antibody purification)  
80.2 ml 1M \( \text{K}_2\text{HPO}_4 \)  
19.8 ml 1 M \( \text{KH}_2\text{PO}_4 \)  
add water to give 1000 ml

2.2 Methods

2.2.1 Resistance and paracellular flux measurements

Resistance measurements of cells grown on 6.5 mm transwells were taken using a Millicell-ERS resistance system. For paracellular flux measurements, 1 \( \mu \text{Ci} \) of \([\text{U-}^{14}\text{C}]\text{sucrose in 25 \( \mu \text{l} \) PBS} \) was added to the apical side of cells grown on 6.5 mm Transwells. Cells were incubated at 37°C, shaken at 10 minute intervals, and the entire medium from the basolateral chamber was removed for scintillation counting at the times indicated.

2.2.2 Treatment with reagents

EGF, H-7 and calyculin A were made up in PBS. EGF was added to cells at 1:1000, and H-7 and calyculin A were added at 1:100 to give the appropriate final concentrations. All other reagents were made up in DMSO. PD 98059 and DiC₆ were added to cells at 1:100 dilution; all other reagents were added at a dilution of 1:1000 to give the required final concentrations. Where cells were grown on Transwell filters, reagents were added to both the apical and basolateral side of the cells. Vehicle controls were added at appropriate dilutions in each experiment.

2.2.3 Cell Lysis and Immunoprecipitation

Whole cell lysates were prepared by rapidly replacing the culture medium of cells on filters with hot Laemmli sample buffer, followed by heating at 100°C for 5 minutes. Immunoprecipitations were carried out at 4°C in which case, after appropriate treatment, confluent monolayers of cells were washed rapidly with ice-cold PBS
(containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$) before addition of 0.5 ml of Triton or TDS lysis buffer. The filters were then gently scraped, and the lysate was removed and centrifuged at 14,000 g. The supernatant was precleared with 50 μl 10% (w/v) protein A-Sepharose in lysis buffer for 30-60 minutes. Appropriate primary antibodies were added to the lysate (at a concentration of approximately 2.5 μg per 5 x 10$^5$ cells), and after 2-3 hours the immune complex was isolated, using 5 μg rabbit anti-mouse secondary antibodies where mouse primary antibodies had been used, and 50 μl 10% protein A Sepharose. The beads were washed five times with lysis buffer, and the bound protein was solubilised in Laemmli sample buffer, followed by heating at 100°C for 5 minutes.

Where immunoprecipitations were carried out using proteins in the TDS insoluble fraction, 1 ml of TDS lysis buffer was added to a 9 cm dish of confluent MDCK I cells (after appropriate treatment), the cells were scraped, and the insoluble fraction obtained by centrifugation at 14,000 g. The pellet was resuspended in 0.2 ml SDS lysis buffer, by repeated passage through a 23 gauge syringe needle. The sample was heated at 100°C for 3 minutes, and then mixed with 0.8 ml of Triton dilution buffer. Immunoprecipitations were carried out at 4°C as described above.

2.2.4 Electrophoresis and Immunoblotting

Cell extracts or immunoprecipitates in SDS sample buffer were resolved by SDS-PAGE (Laemmli, 1970). Resolving gels (1.5 mm or 0.75 mm thick) were made in 0.375 M Tris-HCl (pH 8.8), and 0.1 % SDS, with acrylamide-bisacrylamide added to give a final percentage of 6 or 8 % (w/v) as required. Polymerisation was achieved using ammonium persulphate (0.05% w/v) and Temed (0.03% v/v). The mixture was poured between two glass plates, to within 4 cm of the top. This was overlaid with water-saturated isobutanol until polymerised. Stacking gel, consisting of 4% acrylamide-bisacrylamide, 0.1% ammonium persulphate, 0.05% Temed, 0.1% SDS in 0.125 M Tris-HCl (pH 6.8), was poured on top of the resolving gel, a 12-well comb inserted, and the gel left to polymerise for 30 minutes at room temperature. Separation
was carried out overnight at 50 V, using the Hoefer electrophoresis system. For transfers, the slab gels were equilibrated in transfer buffer for 30 minutes. Proteins were then transferred to nitrocellulose filters or Immobilon P, using the BioRad Trans-Blot cell system (50 V for two hours, high field, at 4°C). Filters were stained with Ponceau S for 10 minutes, destained with deionised water and air-dried. Non-specific binding sites were blocked by incubating filters for 2 hours at 37°C with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20. Filters were then probed with primary antibody for 60 minutes at room temperature or overnight at 4°C, at appropriate dilutions (see below). After washing, filters were incubated with horseradish peroxidase-conjugated anti-mouse antibody, at a dilution of 1:5000 in 1% BSA/PBS/0.05% Tween-20. The filters were then extensively washed in PBS/0.5% Tween-20, and immunoreactive bands were detected by enhanced chemiluminescence (Amersham) following the manufacturers instructions. Primary antibody dilutions (unless indicated otherwise) were: pp120 and β-catenin, 1:5000; α-catenin, E-cadherin, paxillin, RC20H, 1:2500; GSK-3β, Ras, Erk-2, phosphoERK, ZO-1, 1:1000; pepZO-2, ZO-2 1:500. Where blots were reprobed, stripping was carried out in 0.1 M glycine (pH 2.5) at room temperature for 20 minutes, washed extensively in PBS, then blocked in 1% bovine serum albumin in PBS-Tween and reprobed with antibodies.

2.2.5 Phosphate Labelling

MDCK I, EA.hy926 or NIH 3T3 cells grown to confluence on 24 mm filters or plastic dishes were incubated overnight in phosphate-free medium, containing 0.5% serum (dialysed against 0.9% NaCl and 10 mM Hepes (pH 7.5)) and 100 μCi/ml [³²P]orthophosphate. Cells were treated with or without 200 nM PDB for 30 minutes, then were lysed into TDS buffer, and immunoprecipitated with antibodies as described above. Following transfer of proteins to nitrocellulose, the filters were exposed overnight to film, and the resulting autoradiograph of [³²P]labelling was scanned by densitometry using a BioRad Imaging Densitometer GS-670. The filters were then
probed with appropriate antibodies, and the resulting ECL exposure scanned in the same way, to quantify protein loading.

2.2.6 Phosphoamino acid (PAA) analysis

MDCK I cells were labelled as above with \(^{32}\text{P}\)orthophosphate, and specific adherens junction proteins extracted from PDB-treated or untreated cells by immunoprecipitation. Following transfer to Immobilon P, and immunoblotting to assess recovery of protein, the area of the filter containing phosphorylation protein was excised. The proteins were hydrolysed at 110°C for one hour in 5.7 M HCl to release PAAs. Following lyophilisation, the PAAs were dissolved in 10 \(\mu\)l pH 1.9 buffer containing phosphoserine, phosphothreonine and phosphotyrosine at final concentrations of 0.07 mg/ml. Two dimensional PAA separation and detection was carried out as previously described (Boyle et al., 1991); PAAs were spotted onto 20 cm\(^2\) cellulose plates, which were wetted in pH 1.9 buffer, and electrophoresed at 1.5 kV for 20 minutes. Markers (1 \(\mu\)l of \(\epsilon\)-DNP-lysine and xylol cyanol FF (5 mg/ml in pH 1.9 buffer) were spotted along with the PAAs to track the progress of the electrophoresis. Plates were air-dried for 60 minutes, then wetted in pH 3.5 buffer, and electrophoresed in a perpendicular direction at 1.3 kV for 16 minutes. Plates were dried, then sprayed with 0.25% ninhydrin in acetone, and baked at 65°C for 20 minutes, to visualise the cold phosphoamino acid standards. Plates were then exposed to film at -80°C using intensifying screens.

2.2.7 Phosphopeptide mapping

\(^{32}\text{P}\)-labelled p100 was immunoprecipitated as described above. Following gel electrophoresis, gels were fixed for 60 minutes in water:methanol:acetic acid at a ratio of 65:25:10. The gel was dried (BioRad Model 583 gel drier), and the protein detected by autoradiography, and excised. Protein was purified by a modification of the method described by Boyle et al.. Gel slices were homogenised using a Kontes disposable plastic pestle in 0.4 ml of 50 mM NH\(_4\)HCO\(_3\) (pH 7.4) with 0.1% w/v SDS. The pestle
was washed with 0.8 ml buffer, bringing the total volume to 1.2 ml. 12 μl of β-mercaptoethanol and 6 μl of 20 % SDS was added to the tube, the contents vortexed and heated at 100°C for 3 minutes. The tubes were then capped and rotated at room temperature overnight. The gel fragments were separated from the supernatant by spinning for 5 seconds in a Spin-X tube. The flow-through was collected. Protein was precipitated by adding 0.3 ml trichloroacetic acid, and 10 μg of gamma globulin as a carrier, and incubating on ice for 2 hours. The samples were centrifuged at 14,000 g at 4°C for 10 minutes, the supernatant removed, and the pellet dried by washing in ethanol, followed by air drying. 20 μl of performic acid (made fresh by mixing 0.1 ml formic acid with 0.01 ml H₂O₂ at room temperature for 60 minutes) was added to the protein pellet for 1 hour on ice. This was to oxidise the protein. The reaction was quenched with 0.4 ml water, and the sample lyophilised. The protein was digested for 16 hours at 37°C with either 10 μg trypsin or thermolysin in 50 μl of 50 mM NH₄HCO₃. Another 10 μg of protease was added, and incubated for a further 4 hours at 37°C. 0.4 ml deionised water was added, and the sample heated at 70°C for 5 minutes, then centrifuged at 13,000 rpm for 10 minutes in a microfuge. The sample was lyophilised, dissolved in water, lyophilised again, and then dissolved in 20 μl water.

Peptide was spotted onto a cellulose plate in 1 μl amounts. 20 x 20 cm plates were used, and the peptide spotted at a point 4 cm from one edge and midway between the two perpendicular edges of the plate. Tracker dye (1 μl of ε-DNP-lysine and xylol cyanol FF (5 mg/ml in electrophoresis buffer)) were also spotted here. The plate was wetted in pH 3.5 buffer using Whatman 3MM filter paper soaked in buffer with a hole cut over the region where the peptide had been spotted. Electrophoresis was then carried out at 1.2 kV for 16 minutes. Plates were air dried, and then chromatography carried out a right angles to the direction of electrophoresis in phosphochromatography buffer (n-butanol: pyridine: glacial acetic acid: deionised water in a ratio of 15:10:3:12), or isobutyric acid buffer (isobutyric acid: n-butanol: pyridine: glacial acetic acid:
deionised water at 1250:38:96:58:558) until the buffer front was 2 cm from the top of the plate. Plates were then dried and exposed to film at -80°C.

2.2.8 [35S]methionine labelling of p120 associated proteins

MDCK I cells were washed twice in methionine-free MEM supplemented with 0.5 % FCS. Cells were then incubated for 48 hours in 50 μCi/ml [35S]methionine (>1000 Ci/ mmol) in methionine-free MEM prior to immunoprecipitation. p100/p120 and associated proteins were isolated by immunoprecipitation in triton lysis buffer. Protein analysis was by SDS-PAGE, followed by fixation in 25 % methanol/10 % acetic acid. Labelled protein was detected by fluorography at -80°C following impregnation of the gel with Amplify™ for 20 minutes.

2.2.9 Protein stability determination

Pulse-chase analysis of p100/p120 to determine protein stability was carried out as follows: MDCK I cells labelled overnight in methionine-free MEM, 0.5% FCS and 50 μCi/ml [35S]methionine were washed three times in normal medium, then incubated in the presence of 200 nM PDB or DMSO at 1:1000 as a control. Cells were extracted into TDS lysis buffer at various time points, the p100/p120 purified by immunoprecipitation, and visualised by fluorography of SDS-PAGE gels fixed and equilibrated in Amplify™ as above.

2.2.10 In vitro kinase assays

All in vitro kinase assays were carried out in kinase assay (KA) buffer (see stock solutions above). For analysis of phospho-GS-peptide phosphorylation by recombinant GSK-3β, 25 or 50 mUnits of enzyme was mixed with 5 μg of phospho-GS-peptide (or phospho[Ala21]-GS-peptide as a control) in 20 μl of KA buffer. Kinase inhibitors were added in 1 μl DMSO to give the required final concentration, and incubated for 10 minutes on ice. The kinase reaction was started by addition of 10 μl of KA buffer containing 5 μM cold ATP, and 1 μl [γ-32P]ATP (250 μCi/ml), and the
reaction was incubated at 30°C for 10 minutes. The reaction was spotted on to 2 cm x 2 cm squares of P81 chromatography paper, which were washed extensively in 0.75% \( \text{H}_3\text{PO}_4 \). The squares were washed once in acetone, and dried, and the incorporation of radioactivity was assayed by scintillation counting. In cases where GSK-3β was purified by immunoprecipitation from MDCK I cells, the protein A Sepharose pellet, with bound protein, was washed in KA buffer then resuspended in 20 μl with kinase inhibitors and the reaction carried out as above.

*In vitro* phosphorylation of β-catenin and p120 was carried out in KA buffer. p100/p120 and β-catenin were immunoprecipitated in TDS lysis buffer. Following washing, the protein bound to protein A Sepharose was washed once in KA buffer, and resuspended in 20 μl of buffer. 50 mUnits of GSK-3β enzyme were added, and the reaction started by addition of 10 μl of kinase assay buffer containing 5 μM cold ATP, and 1 μl [γ-\(^{32}\)P]ATP (250 μCi/ml). The reaction was carried out at 30°C for 10 minutes, and quenched by addition of 2 x Laemmli sample buffer. Protein were separated by SDS-PAGE, transferred to nitrocellulose, and \(^{32}\)Pphosphate incorporation measured byautoradiography. The filter was then probed with antibodies to β-catenin or p100/p120 to assess the recovery of protein.

### 2.2.11 Immunofluorescence

Cells grown on either 6.5 mm polycarbonate filters or 13 mm diameter coverslips were fixed at room temperature for 20 minutes (or 3 minutes in the case of pepZO-2 staining) in 3% paraformaldehyde made up in PBS containing 0.5 mM CaCl₂ and 0.5 mM MgSO₄. Fixed cells were washed and then permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 minutes. After washing, the cells were incubated for 30 minutes in immunofluorescence “block”. Incubation with antibody was in “block” for 1 hour. Antibody concentrations were; β-catenin and p120, 0.5 μg/ml; pepZO-2, E-cadherin and α-catenin 2.5 μg/ml. After washing, the cells were then incubated for 60 minutes with a 1:100 dilution of FITC-conjugated anti-mouse IgG or FITC-conjugated anti-rabbit antibody in “block”. In some instances, the actin
cytoskeleton was labelled using rhodamine-labelled phalloidin added with the secondary antibody to a final concentration of 2 Units/ml. After washing, the filters were mounted with Citifluor and examined using a Nikon Microphot-FXA fluorescence microscope fitted with 40 x and 100 x objectives. Photographs were taken using Kodak T-MAX film (400 ASA).

2.2.12 Peptide-directed antibody generation

Peptides corresponding to amino acids 889-901, (M13K) and 966-978 (S14K) of ZO-2, with an extra lysine not present in the protein added to the C-terminal end of the peptide, were synthesised by ImmuneSystems Ltd., Bristol, UK, and stored at -20°C. Peptides were coupled to KLH with glutaraldehyde as follows: 10 mg of peptide was dissolved in 1 ml PBS, and the pH adjusted to 7.5; 20 mg of KLH dissolved in 1 ml of PBS was then added to the peptide solution, followed by addition of 2 ml of 0.2% glutaraldehyde in PBS, added in a dropwise manner with constant mixing. After one hour, 1 ml of 1 M glycine in PBS was added, and the solution rotated at room temperature for another hour. For immunisation, 1 ml of M13K peptide-KLH solution was mixed with 1 ml S14K peptide-KLH, and added to the contents of one vial of Ribi adjuvant, and the solution vortexed for 4 minutes. This mixture was used to immunise two rabbits (1 ml per rabbit) at Froxfield Farms, UK.

After several rounds of intradermal and subcutaneous immunisations at intervals of three weeks, specific antibodies were isolated on peptide-e-carboxyhexanol (ECH)-sepharose columns. Coupling of peptides to ECH-sepharose columns was as follows: 10 mg each of M13K and S14K peptide was dissolved in 1 ml water (pH 4.5) and the pH readjusted to 4.5 The peptide solution was mixed with 1 ml EDC-carbodiimide (100 mg/ml, pH 4.5, and loaded onto 1 ml ECH-sepharose columns, which had been washed with 80 ml of 0.5 M NaCl, then 50 ml of water (pH 4.5). The column mixture was rotated overnight at room temperature. The column was then drained, and washed with 10 ml water (pH 4.5). Remaining carboxy groups were blocked by incubation of the column with 1 ml ethanolamine (0.4 M, pH 4.5) at room temperature for two hours,
with constant mixing by rotation of the column. The column was then washed with 50 ml water (pH 4.5), then 10 ml of KPi buffer and then 10 ml of glycine-HCl (0.1M, pH 2.5). Columns were stored at 4°C in PBS containing 0.02% azide.

For affinity purification of antibodies the columns were washed with 10 ml KPi buffer, and then incubated with 2 ml serum (1 ml from each immunised rabbit), and incubated at 4°C overnight, with mixing by rotation. The columns were washed with 50 ml KPi buffer at 4°C, and then bound antibody was eluted by adding 3 ml of 0.1 M glycine (pH 2.5). This was neutralised by addition of 70 µl 0.75 M Tris base (pH 8.8) immediately after elution. Antibodies were stored in aliquots at -20°C.
3. Phosphorylation state of cadherins, catenins and p120$^{ca}$ isoforms in response to activation of PKC in epithelial cells

3.1 Introduction

MDCK I cells provide a good epithelial cell system in which to study the integrity of tight junctions. They are derived from canine kidney, and monolayers develop an extremely high resistance due to the presence of well-developed tight junctions which provide an extremely effective barrier to transport of ions between cells (Richardson et al., 1981). Agents which disrupt tight junctions will cause a drop in resistance, which can easily be measured in cell layers grown on porous Transwell filters.

Activation of protein kinase C (PKC) is known to cause disruption of tight junctions in number of different epithelia. Proteins of the PKC family promote the phosphorylation of a wide range of proteins. Thus, activation of PKC might regulate adhesion by phosphorylation of one or more components of intercellular junctions. The presence of an intact adherens junction is vital for the formation and maintenance of other intercellular junctions, including the tight junctions. Therefore, I investigated whether components of the adherens junction are targets for PKC.

3.2 Characterisation of the effects of phorbol ester on tight junctions in MDCK I cells

To confirm that activation of PKC promoted the disruption of tight junctions in MDCK I cells (Ojakian, 1981), the effect of the phorbol ester phorbol-12,13-dibutyrate (PDB) on the paracellular permeability of MDCK I cell monolayers was characterised. MDCK I layers with an initial resistance of 2000-4000 $\Omega$.cm$^2$ were treated with 200 nM PDB, or vehicle DMSO as a control, and the resistance across the monolayer was measured over time. A typical experiment is shown in fig. 3.1A.
Fig. 3.1 MDCK I cell monolayer permeability is increased in response to activation of PKC

(A) Electrical resistance decreases rapidly on addition of 200 nM PDB. The vehicle DMSO has no effect on resistance. (B) Paracellular flux of $[^{14}\text{C}]$sucrose increases following addition of 200 nM PDB. In both A and B, each point represents the mean of data from triplicate Transwells, with standard deviation indicated by error bars. Where there are no error bars, standard deviations are too small to be visible.
Although a drop in resistance is consistent with disruption of tight junctions, other physiological processes can also cause an apparent decrease in resistance across cell monolayers. One example is opening of Cl channels, which allows anions to flow transcellularly, rather than paracellularly. To check that the resistance drop seen in MDCK I cells in response to PKC was due to disruption of tight junctions, and not an effect on Cl channels, the flux of [14C]sucrose was assayed. This tracer molecule can only pass through cells via the paracellular pathway. Addition of 200 nM PDB led to an increase in sucrose flux from the apical to the basolateral chamber (fig. 3.1 B), showing that activation of PKC did indeed cause disruption of the paracellular tight junction barrier in MDCK I cells.

3.3 Phosphorylation of the cadherin/catenin complex

The phosphorylation state of adherens junction proteins was investigated after 30 minutes of stimulation with PDB. At this time point, resistance across the MDCK I monolayer has dropped significantly (averaging results from four separate experiments showed that resistance across MDCK I monolayers dropped to 58±7.7% of control), but resistance still continued to decrease. Thus, 30 minutes marks a time point midway through the process by which intercellular junctions are disrupted in response to activation of PKC. In order to investigate whether the phosphorylation state of adherens junction proteins was altered in cells treated with PDB, the phosphate pool was labelled by overnight incubation with [32P]orthophosphate. Cells were treated with PDB, or DMSO as a control, for 30 minutes. Cells were lysed into Triton lysis buffer, and β-catenin was extracted by immunoprecipitation. Under these lysis conditions, protein-protein interactions are maintained, so E-cadherin, p100, p120 and α-catenin were also recovered by immunoprecipitation with β-catenin antibodies. Phosphate content was analysed by autoradiography. Following treatment with PDB, the phosphate content of the cadherin/catenin complex decreased (fig. 3.2 A). This effect appeared to be specific for the cadherin/catenin complex. Whole cell extracts from PDB-treated cells showed no global loss of phosphate, although there were differences
Fig. 3.2 Phosphate labelling of cadherin/catenin complex, following activation of PKC.

(A) MDCK I cells were labelled with $[^{32}P]$orthophosphate, treated with 200 nM PDB (+), or DMSO (-), then lysed into triton lysis buffer. Antibodies against $\beta$-catenin were used to immunoprecipitate (IP) the cadherin/catenin complex. The expected mobility of E-cadherin (E), $\alpha$-catenin ($\alpha$), and $\beta$-catenin ($\beta$) are indicated. p100 and p120 migration overlaps that of E-cadherin and the catenins, and is indicated by the regions enclosed in brackets ($\}$).

(B) $\beta$-catenin probe of $^{32}P$-labelled $\beta$-catenin immunoprecipitates, to demonstrate equal protein recovery.

C: $[^{32}P]$phosphate-signal from whole cell triton lysates, treated with (+) or without (-) PDB. The migration of molecular weight standards is indicated. A number of proteins showed increased (○) or decreased (●) phosphorylation in response to PDB.
in the phosphorylation state of some proteins from PDB-treated cells relative to control (fig. 3.2 C).

3.4 Dephosphorylation of p100 and p120

In order to identify which components of the adherens junction became dephosphorylated in response to PDB, each component was analysed separately. A significant change was found to occur to the two p120iso isoforms that are expressed in epithelial cells; p120 and p100.

MDCK I cells were metabolically labelled with [32P]orthophosphate, treated with or without PDB, and the p100 and p120 isolated by immunoprecipitation. This immunoprecipitation was carried out in TDS lysis buffer, to remove any associated proteins from p100 and p120. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and analysed by autoradiography (fig. 3.3 A). The level of phosphate present in p100 and p120 from untreated cells was compared with that from PDB-treated cells by densitometric scanning of the resulting autoradiograph (fig. 3.3 B). Immunoblotting of these filters, followed by scanning, allowed the phosphate signal to be normalised in relation to amount of protein analysed. (fig. 3.3 A and B). This corrected for small errors due to slight differences in protein recovery. It was calculated that the [32P]phosphate content of p100 and p120 was reduced by approximately 40% following PDB treatment.

Phosphoamino acid (PAA) analysis of p100/p120 from MDCK I cells showed that in resting cells these proteins contained mainly phosphoserine, with a small amount of phosphothreonine, and no detectable phosphotyrosine (Fig. 3.3 C). Following addition of PDB, there was a reduction in the level of phosphorylation of both serine and threonine residues. Densitometric analysis of the autoradiographs of the PAA analysis revealed a reduction in phosphate signal of approximately 40%, agreeing with the values obtained from the whole protein phosphate labelling experiments.
Fig. 3.3 Dephosphorylation of p100/p120 following PDB-treatment

(A) Following overnight labelling with $[^{32}\text{P}]$orthophosphate, MDCK I cells were exposed for 30 minutes to 200 nM PDB, or vehicle only, and lysed in TDS buffer. p100 and p120 were immunoprecipitated, blotted and labelled protein was visualised by autoradiography. The level of phosphate in p100 and p120 from control cells, relative to that from PDB-treated cells, was determined by densitometric scanning. To normalise for slight differences in protein loading, the same filters were then probed with p100/p120 antibody, and the level of p100/p120 protein was determined by densitometry. Thus, the amount of phosphate per unit protein could be calculated. Panel B shows the results of the scanning of the autoradiographs shown in panel A, with $^{32}\text{P}$ and protein signal measured in OD.mm.mm. 'Pn' indicates 'phosphate level normalised for protein'. In this experiment, approximately 40% of the phosphate in p100/p120 was lost following PDB treatment. The results from seven independent sets of such experiments showed that following PDB treatment the phosphate content of p100/p120 was reduced to 58 ± 11% (mean ± SD) of that of untreated cells.

(C) phosphoamino acid (PAA) analysis. Following phosphate labelling, immunoprecipitation and visualisation of labelled p100/p120 as above, the levels of p100/p120 protein were determined by immunoblotting, and in this case it was ascertained that equal amounts of protein from PDB-treated and untreated cells were used for the PAA analysis. The p100/p120 bands were excised, and the PAAs were separated by 2-dimensional electrophoresis. p100/p120 from control cells contained mainly phosphoserine (S), some phosphothreonine (T) but no detectable phosphotyrosine (Y). Following PDB treatment, both phosphoserine and phosphothreonine levels were reduced.
A  
Detection:  
32P  
protein  
Set number:  
1  -  +  2  -  +  1  -  +  2  -  +  

97 kDa  
p120  p100  

B  
PDB Treatment:  
32P (OD.mm.mm)  18.7  7.90  17.4  10.8  
Protein (OD.mm.mm)  12.9  9.41  14.7  13.8  
32P/protein (Pn)  1.45  0.84  1.18  0.78  

Pn (Control) : Pn (PDB)  1 : 0.58  1 : 0.66  

C  
Control  
PDB  
S  T  Y  
S  T  Y
3.5 Phosphate content of E-cadherin, β-catenin and α-catenin

E-cadherin, α-catenin or β-catenin were extracted by immunoprecipitation from [32P]orthophosphate-labelled MDCK I cells under TDS lysis conditions. Phosphate signal was analysed by autoradiography, and protein levels were determined by western blot (fig. 3.4). E-cadherin appears to be highly phosphorylated in resting MDCK cells, mainly on serine residues, although a small amount of phosphotyrosine can be detected. β-catenin is also phosphorylated on phosphoserine in untreated and PDB-treated cells. The phosphate signal from immunoprecipitated α-catenin was too faint for a phosphoamino acid analysis to be carried out. It is likely that α-catenin contains very few phosphorylated residues. There was no significant change in the phosphorylation state of any of these proteins following stimulation of PKC with PDB. However, phosphorylation or dephosphorylation of a single site, or a small number of sites may have escaped detection in this assay. Also, any complicated change in phosphorylation pattern, such as a phosphorylation of one site, and concomitant equal dephosphorylation of another site would not be detected.

The only significant change in phosphorylation in response to PDB was the dephosphorylation of p100 and p120. The specificity of this dephosphorylation rules out the extremely unlikely possibility that p100/p120 dephosphorylation was an artefact due to non-specific effects of PDB on labelling of the γ-phosphate of the cellular ATP pool. Since the catenins, p100 and p120 have overlapping mobility in SDS-PAGE, the apparent global dephosphorylation of the cadherin/catenin complex that was seen in Triton lysis conditions (fig. 3.2) could be due to reduction of phosphate signal from p100 and p120. However it is also possible that the phosphatase responsible for the dephosphorylation of p100/p120 remains associated with the adherens junctions complex under Triton lysis conditions, and may promiscuously dephosphorylate all the components of the complex during the immunoprecipitation.
Fig. 3.4. Phosphate labelling of E-cadherin and catenins.

(A) MDCK I cells labelled overnight with $^{32}$P were treated with 200 nM PDB (+) or DMSO as a control (-). Cells were then lysed into TDS lysis buffer, and E-cadherin, β-catenin or α-catenin isolated by immunoprecipitation, separated by SDS-PAGE, and transferred to nitrocellulose. Phosphate content was analysed by autoradiography, and protein content by Western blot. None of these proteins show a significant change in phosphate content in response to PDB.

(B) Phosphoamino acid analysis of E-cadherin and β-catenin, from untreated (-) or PDB-treated (+) MDCK I cells. α-catenin phosphate signal was too weak for a phosphoamino acid analysis to be performed. Position of PAA standards phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated. E-cadherin contains mainly phosphoserine, with a small amount of phosphotyrosine. Only phosphoserine could be detected in β-catenin.
3.6 p100/p120 dephosphorylation visualised in western blots

The dephosphorylation of p100 and p120 caused these proteins to have a markedly altered electrophoretic mobility. Whole cell lysates from MDCK I cells treated with or without 200 nM PDB for 30 minutes were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies recognising p100/p120 (fig. 3.5 A). Whereas the p100 and p120 from untreated cells were visualised as rather diffuse bands, these proteins from PDB-treated cells appeared as tighter, faster migrating bands. In contrast, the mobility and the amount of E-cadherin were unaffected (fig. 3.5 B), indicating equal protein loading, and, as previously reported for fibroblasts (Zachary et al., 1993), the mobility of paxillin was reduced due to phosphorylation (fig. 3.5 C).

3.7 PKC-dependence and reversibility of p100/p120 dephosphorylation

Pre-treatment of MDCK I cells with the PKC inhibitors bisindolylmaleimide I (Toullec et al., 1991) or Ro 31-8425 (Wilkinson et al., 1993) for 5 minutes prior to PDB addition completely abolished the p100/p120 dephosphorylation (fig. 3.6 A). Bisindolylmaleimide V, a member of the bisindolylmaleimide class that does not inhibit PKC (Davis et al., 1992), was unable to block the PDB-induced band shift. Analysis of dose-dependence showed that PDB was effective at concentrations between 30 and 100 nM (fig. 3.6 B), corresponding to the concentrations required to activate PKC (Castagna et al., 1982; Rozengurt et al., 1983). Also, an inactive stereoisomer of PDB, 4α-PDB, had no effect on p100/p120 mobility (fig. 3.6 C). The effect of PDB could be also be obtained with a cell permeant diacylglycerol analogue, 1,2-dioctanoyl-sn-glycerol (DiC8) (fig. 3.6 D). These results demonstrate that the p100/120 band shift occurs as a response to PKC activation by PDB.

A time course experiment showed that the p100/p120 mobility shift occurred rapidly, with maximal effect within 5 minutes of PDB treatment (fig. 3.7 A). The effect on p100/p120 could be reversed, either by washing away the PDB (data not shown), or by subsequent addition of bisindolylmaleimide I. These data show that interconversion
Fig. 3.5. The migration of p100 and p120 during SDS-PAGE is increased following PDB treatment of MDCK I cells.

Cells treated for 30 minutes with either 200 nM PDB or vehicle (DMSO at a 1:1000 dilution) were lysed into Laemmli sample buffer and separated by SDS-PAGE using 6% polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with antibodies to (A) p100/p120, and then reprobed with antibodies recognising either (B) E-cadherin or (C) paxillin. PDB treatment caused p100 and p120 to migrate as faster, tighter bands, had no effect on E-cadherin, but retarded the mobility of paxillin.
Fig. 3.6. p100/p120 dephosphorylation is due to activation of PKC.

(A) PKC inhibitors block the PDB-induced p100/p120 band shift. MDCK I cells were pre-treated with either bisindolylmaleimide I (Bis I) at 2.5 μM, an inactive analogue of bisindolylmaleimide I, bisindolylmaleimide V (Bis V) at 2.5 μM, or Ro 31-8425 (Ro) at 5 μM for 5 minutes, and then exposed to 200 nM PDB for 30 minutes. (B) PDB dose dependence. MDCK I cells were incubated for 30 minutes with a range of PDB concentrations from 1 to 200 nM. p100/p120 mobility increased with increasing PDB concentration, with maximal affect at 100 nM PDB. (C) A stereoisomer of PDB, 4α-PDB, which does not activate PKC, has no effect on p100/p120 mobility. 4α-PDB was added to give a final concentration of 200 nM for 30 minutes. (D) DiC8, a cell permeant diacylglycerol analogue, induced a p100/p120 mobility shift. MDCK I cells were treated with 0.5 mM DiC8 for 10 minutes. In all cases, cell lysates were analysed by SDS-PAGE on 6% polyacrylamide gels, and immunoblotted with p100/p120 antibodies. The migration of p100 and p120 is indicated.
Fig. 3.7. Time dependence and reversibility of the PDB effect.
MDCK I cells were treated with 200 nM PDB for the times indicated, and p100/p120 detected by immuoblot (A). Reversibility was demonstrated by initially treating cells with 200 nM PDB for 30 minutes, and then adding 2.5 μM bisindolylmaleimide I (still in the presence of PDB) for the times indicated. Maximal effect of PDB on p100/p120 was seen between 1 and 5 minutes, and could be reversed by bisindolylmaleimide I. The resistance of the MDCK I monolayers correlates closely with the p100/p120 band shift (B), with a rapid resistance drop after addition of PDB, followed by an equally rapid re-establishment of resistance after inhibition of PKC by bisindolylmaleimide I. Cells were treated with 2.5 μM bisindolylmaleimide I alone (□), 200 nM PDB alone (○) or 200 nM PDB with 2.5 μM bisindolylmaleimide I added after 30 minutes (●).
of p100 and p120 from slower to faster migrating forms is dynamic, clearly arguing against the possibility that the increased p100/p120 mobility was due to proteolysis. In these cells the mobility shift of p100 and p120 correlates closely with the kinetics of permeability increase across the cell monolayer, and reversal of the shift by addition of bisindolylmaleimide I is rapidly followed by re-establishment of monolayer resistance (fig. 3.7 B).

3.8 Effect of staurosporine and phosphatase inhibitors on p100 and p120

In order to bring about dephosphorylation of p100/p120, PKC activation must lead to net kinase inhibition and/or phosphatase activation. Since the effect on p100/p120 is rapid and can easily be reversed, it is clear that p100/p120 is capable of cycling between phosphorylated and lesser phosphorylated forms, subject to the action of kinase(s) and phosphatase(s). In order to test this further, MDCK I cells were treated with kinase or phosphatase inhibitors to try and perturb this cycle. Staurosporine, a kinase inhibitor (Tamaoki et al., 1986), at 100 nM induced a p100/p120 band shift similar to that seen in response to PDB (fig. 3.8 A). This would be consistent with direct inhibition of a p100/p120 kinase by staurosporine.

Addition of the serine/threonine phosphatase inhibitors cantharadin, calyculin A and okadaic acid had the opposite effect on p100/p120 mobility: induction of an upward band shift, consistent with increased phosphorylation (fig. 3.8 B). Thus it would appear that in resting cells, a certain level of p100/p120 phosphorylation is maintained by a balance of the opposing effects of kinase and phosphatase. Inhibition of the kinase shifts the balance in favour of the phosphatase, causing net p100/p120 dephosphorylation. In contrast, inhibition of the phosphatase leads to predominant kinase activity, manifested by increased phosphorylation of p100/p120. Cantharadin, calyculin A and okadaic acid inhibit protein phosphatases 1 and 2A (Li and Casida, 1992; Shenolikar, 1994). Cypermethrin, which inhibits protein phosphatase 2B (Enan and Matsumura, 1992) had no effect on p100/p120 mobility. Thus it appears that p100
Fig. 3.8. Effect of staurosporine and phosphatase inhibitors on p100/p120 mobility.

(A) Addition of the broad range kinase inhibitor staurosporine (100 nM for 30 minutes) to MDCK I monolayers induces dephosphorylation of p100 and p120.

(B) Addition of the phosphatase inhibitors okadaic acid (1 μM), calyculin A (100 nM), or cantharadin (40 μM) for 60 minutes caused an upward band shift of p100/p120, consistent with increased phosphate content. Cypermethrin at 100 nM had no effect on p100/p120.
and p120 are substrates for protein phosphatases 1 and 2A, but not 2B. However, it is not possible to conclude that PP 1 and PP 2A these are the phosphatases responsible for the dephosphorylation of p100 and p120 in response to PKC activation.

3.9 p100/p120 dephosphorylation in various epithelia

The mobility of p100 and p120 was assayed in a number of epithelial cell lines. Cell lines used were; MDCK II, an MDCK I-derived cell line from canine kidney; Caco-2, a human intestinal carcinoma cell line; LLC-PK₁, a porcine kidney cell line; MDBK, a bovine kidney derived cell line and SAEC, primary human small airway epithelial cells. In all these cells, p100 and p120 showed the same kind of increase in mobility in response to PKC activation (fig. 3.9), although the magnitude of the effect varies between different cell types. p100/p120 from LLC-PK₁ and MDCK II cells show a striking alteration in mobility in response to PDB. The mobility shift also occurs in MDBK, SAEC and Caco-2 cells, but is less pronounced. p100 and p120 were found to be targets for a PKC-mediated signalling pathway every epithelial cell line tested. It is possible that this pathway is a characteristic of all epithelial cells, and therefore could be of fundamental importance in epithelial biology.

3.10 Discussion

Activation of PKC has been shown to increase the permeability of tight junctions in established epithelial cell monolayers. As adherens junction are necessary for the maintenance tight junction integrity, I investigated whether components of the adherens junction are targets of PKC signalling pathways. Phosphate labelling analysis showed that in resting cells, E-cadherin is phosphorylated primarily on serine residues with some phosphotyrosine. β-catenin is also phosphorylated on serine residues in resting cells. There is no observable alteration in phosphate content of E-cadherin and β-catenin following activation of PKC. Likewise there is no change in the (very low) level of phosphorylation of α-catenin in response to activation of PKC. These
Fig. 3.9. PKC activation induces a p100/p120 mobility shift in a number of different epithelial cell lines.
Cell monolayers were treated with 200 nM PDB for 30 minutes, lysed in to sample buffer and protein separated by SDS-PAGE. p100/p120 mobility was visualised by western blotting. Duplicate experiments (control (-) or PDB (+)) are shown. Cell lines used were; Caco-2 (human intestinal carcinoma), LLC-PK₁ (porcine kidney), MDCK II (canine kidney), MDBK (bovine kidney) and SAEC (primary human small airway epithelial cells).
observations were confirmed by densitometric scanning of the autoradiographs (data not shown).

In contrast, the adherens junction proteins p100 and p120 are targets for a PKC-mediated signal transduction pathway. In unstimulated MDCK I cells, p100 and p120 are phosphorylated primarily on serine residues, with some phosphothreonine, and no detectable phosphotyrosine. $[^{32}\text{P}]$phosphate labelling demonstrated that a significant proportion of these residues are dephosphorylated within 5 minutes of addition of PDB to cells. This loss of phosphate is presumably sufficient to dramatically alter the mobility of these proteins during SDS-PAGE. The dephosphorylation can be reversed rapidly by subsequent inhibition of PKC by bisindolylmaleimide I. These data suggest that in resting epithelial cells, the level of p100/p120 phosphorylation is kept constant by the opposing actions of a serine/threonine phosphatase and a corresponding kinase (see fig. 3.10). Upon addition of PDB, PKC becomes activated, and this must cause inhibition of the p100/p120 kinase and/or stimulation of the phosphatase, leading to net reduction of the phosphate content of p100/p120. Further evidence supporting this idea comes from the effects of kinase and phosphatase inhibitors on p100 and p120 phosphorylation. The broad range kinase inhibitor staurosporine caused faster migration of p100 and p120 during SDS-PAGE, in a similar fashion to that produced by stimulation with PDB. Thus it is possible that the p100/p120 kinase can be directly inhibited by staurosporine, leading to net dephosphorylation of p100 and p120. Conversely, addition of the serine/threonine phosphatase inhibitors cantharadin, okadaic acid and calyculin A caused p100 and p120 to migrate more slowly during SDS-PAGE, consistent with net gain of phosphate. The identification of a p100/p120 serine/threonine phosphorylation cycle contrasts with the previously elucidated pathways by which p120 becomes phosphorylated on tyrosine in response to src over-expression (Kanner et al., 1991), or growth factors such as EGF, PDGF and CSF-1 (Downing and Reynolds, 1991)(fig. 3.10). Indeed, p120 was originally discovered as a tyrosine kinase substrate.
Figure 3.10. Schematic representation illustrating how PKC activation may lead to dephosphorylation of p120

Note that the closely related p120 isoform, p100, would be subject to the same cycle. In resting cells, p120 is phosphorylated on serine and threonine, the level of phosphate being maintained by the opposing actions of a serine/threonine kinase and a phosphatase. PDB activation of PKC may cause it to directly phosphorylate the p120 kinase, switching it from an active (kinase*) form to an inactive (kinase) form as represented here. This would lead to p120 dephosphorylation. Equally, PKC could act to inhibit the p120 kinase via a multi-step signaling pathway. Alternatively, PKC could somehow increase the activity of the serine/threonine phosphatase, which would also cause dephosphorylation of p120. This contrasts with the previously known tyrosine phosphorylation cycle of p120; in resting cells there is little or no tyrosine phosphorylation of p100/p120, but following stimulation of src, or perhaps other tyrosine kinases, p120 is phosphorylated on tyrosine.
It is evident that PKC must be exerting its effects on p100/p120 via a signalling pathway which comprises at least two steps. PKC may act to directly inhibit the p100/p120 kinase, or trigger a multi-step signalling pathway leading to its inactivation. Alternatively, or additionally, PKC may cause p100/p120 dephosphorylation by stimulating phosphatase activity.

The mobility shift of p100 and p120, consistent with dephosphorylation, occurred in response to PDB treatment in a variety of epithelial cell lines, which suggests this PKC-triggered dephosphorylation pathway could be a general feature of epithelial cells. The magnitude of the effect varied between different cell types; a clear mobility shift was seen in MDCK I, MDCK II, MDBK, LLC-PK1, and SAEC cells, but a relatively small change in p100/p120 mobility occurred in response to PDB in Caco-2 cells. However, the p120 isoforms from untreated Caco-2 cells already migrate as rather tight bands during electrophoresis. Perhaps these proteins already exist in a relatively dephosphorylated form, possibly explaining the less striking effect of PDB treatment.
4. Mechanism and nature of p100/p120 dephosphorylation

4.1 Introduction

In order to determine the physiological role of p100/p120 dephosphorylation, it is necessary to find out more about the players involved in this process. Identification of the kinase(s) and phosphatase(s) involved might allow selective manipulation of p100/p120 phosphorylation. I took two approaches to this problem; investigation of the sites of dephosphorylation by phosphopeptide mapping of p100, and investigating potential signalling pathways by which PKC might affect p100/p120 phosphorylation. PKC activates a number of different signalling pathways in cells, any of which could led to inhibition of a p100/p120 kinase and/or activation of a phosphatase. I examined two potential pathways; GSK-3β inhibition, and the MAP kinase pathway.

Glycogen synthase kinase-3 (GSK-3) was first identified as a protein kinase involved in the metabolic pathway by which glucose is converted to glycogen. Molecular cloning revealed the existence of two isoforms, α and β. (Woodgett, 1990; Hughes et al., 1992). Recently, GSK-3β has been shown to be important in the Wnt signalling pathway in vertebrates, and the homologous Wingless pathway in Drosophila (for reviews see Nusse, 1997; Kuhl and Wedlich, 1997). In the absence of Wnt or Wingless signal, GSK-3β phosphorylates β-catenin, targeting it for degradation. Activation of the Wnt/Wingless signalling pathway leads to inhibition of GSK-3β, and subsequent dephosphorylation (and stabilisation) of β-catenin. p100 and p120, like β-catenin and its Drosophila homologue Armadillo, are members of the Armadillo family, and thus share sequence homology. PKC can phosphorylate and inhibit GSK-3β in vitro (Goode et al., 1992). It has also been shown to be involved in the pathway by which the wingless glycoprotein causes inhibition of GSK-3β in 10T1/2 fibroblasts.
(Cook et al., 1996). This raises the possibility that the effect of PKC activation on p100/p120 phosphorylation could be achieved by inactivation of GSK-3β.

MAP kinase pathways are involved in a wide variety of signalling processes. MAP kinases are serine/threonine kinases that are themselves activated by MAP kinase kinases (MAPKKs). These in turn are activated by MAPKK kinases (MAPKKKs). A variety of growth factors, mitogens and cytokines leads to activation of this kinase cascade, and the MAP kinases phosphorylate a wide range of targets (for review see Davis, 1993). PKC can activate the MAP kinase pathway (Howe et al., 1992), and so it is possible that p100/p120 dephosphorylation may be brought about by activation of a MAP kinase pathway by PKC.

4.2 Phosphopeptide mapping of p100

The major p120isoform isoform expressed in MDCK I cells is the p100 protein. Therefore this protein was used for a phosphopeptide analysis. p100 labelled by overnight incubation with [32P]orthophosphate was purified from DMSO or PDB-treated MDCK I cells by immunoprecipitation in TDS lysis buffer. The protein was excised from SDS gels, and recovered by trichloroacetic acid precipitation. p100 was then digested with trypsin, or thermolysin, to generate phosphopeptides. These phosphopeptides were then spotted on to cellulose plates, and separated by electrophoresis in one dimension, followed by chromatography in a perpendicular direction.

The result of a trypsin digestion phosphopeptide map is shown in fig. 4.1. Electrophoretic separation was carried out at pH 3.5, followed by chromatography in buffer containing butanol and pyridine in acetic acid. The pattern of phosphorylation is extremely complicated. If a peptide contains one phosphorylated residue, which becomes dephosphorylated in response to PDB, then a spot that is present on the control DMSO peptide map would be missing from the map generated from PDB-treated cells. If a peptide is phosphorylated on more than one serine/threonine residue
Fig. 4.1 Trypsin phosphopeptide map of p100 from DMSO and PDB-treated MDCK I cells.

MDCK I cells were metabolically labelled with $^{32}$Porthophosphate for 16 hours, treated with DMSO or 200 nM PDB for 30 minutes, then lysed into TDS lysis buffer. p100 was purified by immunoprecipitation and SDS-PAGE, followed by excision from the gel, and precipitation with trichloroacetic acid. The protein was digested to completion with trypsin, and then spotted onto 20 cm$^2$ cellulose plates. Separation was carried out by electrophoresis at 500 V for 60 minutes in buffer containing acetic acid:pyridine:water in a 19:1:89 ratio, with a pH of 3.5. The plates were then rotated through 90 degrees, and chromatography carried out in pyridine:butanol:acetic acid:water in ratio of 15:10:3:12, until the buffer front was with 2 cm of the top of the plate. Plates were dried, and exposed to film. The direction of electrophoresis (E) and chromatography (C) are indicated.
in resting cells, and only one site becomes dephosphorylated, this will be more difficult to detect; the spot will not disappear, although its migration might change. p100 is clearly phosphorylated on a large number of residues. This makes interpretation of the results very difficult, since there are a large number of spots which in some cases overlap. There are some differences in the pattern of phosphopeptides, but it is difficult to identify discrete spots that are lost on addition of PDB. There was also an apparent global decrease in the intensity of phosphorylation of p100 following PDB treatment.

A number of different digestion conditions, as well as a range of chromatographic separation buffers were used in an attempt to gather more information as to the nature of p100 dephosphorylation. The result of a thermolysin digestion, with an isobutyric acid-containing chromatography separation buffer used in the second dimension is shown in fig. 4.2. Again there were some differences in phosphorylation pattern, but it is difficult to identify specific sites of dephosphorylation, and again there was a general decrease in intensity of the phosphopeptide signals. Similar results were seen in different mapping conditions (data not shown). This reduction in intensity of the phosphopeptide spots might be due to a large number of sites become dephosphorylated in p100 in response to PDB. In conclusion, the phosphopeptide mapping approach was not successful in determining the sites of dephosphorylation in response to PDB.

### 4.3 Dephosphorylation of a p100/p120 breakdown product

A serendipitous discovery furnished some information concerning the sites of p100/p120 dephosphorylation. During the immunoprecipitation of 32P-labelled p100/p120 for phosphopeptide analysis, occasional degradation of p100/p120 occurred. This led to the generation of protein fragments with an apparent molecular weight of about 70 kD. These fragments were recognised by the Transduction laboratory pp120 antibody, which recognises an epitope towards the extreme C-terminus of p100 and p120 (fig. 4.3 A, B). These protein fragments showed a loss
Fig. 4.2. Thermolysin phosphopeptide map of p100 from DMSO and PDB-treated MDCK I cells.

\(^{32}\text{P}\)-labelled p100 from DMSO-treated or PDB-treated cells was purified as for the trypsin phosphopeptide map (see legend to fig. 4.1), and digested with thermolysin. Phosphopeptides were spotted onto cellulose plates, and separated by electrophoresis under the same conditions as for the trypsin digest. Chromatography in the second dimension was carried out in buffer comprising isobutyric acid:\(n\)-butanol:pyridine:acetic acid:water in a ratio of 1250:38:96:58:558. The direction of electrophoresis (E) and chromatography (C) are indicated.
of phosphate in response to PDB as well as increased electrophoretic mobility, similar to that seen for the full length p100 and p120. Thus it is likely that these fragments contain the phosphoserine/threonine residues which become dephosphorylated in response to PDB, which suggests that the dephosphorylation sites are in the C-terminal portion of the p100/p120 protein.

p120 differs from p100 by the presence of an additional N-terminal region. Both p100 and p120 exist in at least two isoforms, which differ in the presence (A isoform) or absence (B isoform) of a 21 amino acid sequence spliced in close to the C-terminus (Mo and Reynolds, 1996) (fig. 4.3 C). In MDCK cells, p120A and p120B (also called CAS1A and CAS1B) cannot usually be resolved in SDS-PAGE, and so appear as a single band (termed p120). p100A and p100B (CAS2A and CAS2B) likewise appear as a single p100 protein under normal circumstances. Two fragments, denoted p70a and p70b, were clearly resolved in p100/p120 immunoprecipitates from cells treated with PDB. It is likely that these fragments correspond to truncated versions of the CAS A and CAS B isoforms respectively, which differ in mass by about 3 kD. In the truncated form of p100/p120, these isoforms can now be resolved in SDS-PAGE.

4.4 Selected kinase inhibitors promote dephosphorylation of p100 and p120

The identification of a regulatable serine/threonine phosphorylation cycle (see fig 3.10) predicts that inhibition of the p100/p120 kinase will lead to dephosphorylation of these proteins. A broad range kinase inhibitor, staurosporine, leads to dephosphorylation of p100 and p120, consistent with this hypothesis. MDCK I cells were treated with a number of more restricted kinase inhibitors, and the p100/p120 mobility assayed by western blot (fig. 4.4). The myosin light chain kinase (MLCK) inhibitors KT5926 (Nakanishi et al., 1990; Hashimoto et al., 1991) and ML-7 (Saitoh et al., 1987) caused a p100/p120 mobility shift, consistent with dephosphorylation.
Fig. 4.3. A 70 kD fragment of p120 is dephosphorylated in response to PDB.

MDCK I cells were metabolically labelled with [32P]orthophosphate. p100/p120 was immunoprecipitated under TDS lysis conditions. During this process, fragments with molecular weights of approximately 70 kD were generated, presumably due to some form of protease digestion. The phosphate signal from these fragments was reduced in response to PDB (A). Panel B shows that these fragments are recognised by p120 antibody during immunoblotting. This antibody recognises a region within the C-terminus of p100/p120. Two fragments, denoted p70a and p70b were clearly resolved in cells treated with PDB. It is probable that these fragments correspond to truncated versions of the p120 CAS A and CAS B isoforms respectively (C). The mobility of these fragments is increased in PDB treated cells, in a similar way to that seen for the full length p100 and p120. Thus it is likely that these fragments contain the phosphoserine/threonine residues which become dephosphorylation in response to PDB.
A variety of kinase inhibitors were assayed for their ability to induce a p100/p120 mobility shift on SDS-PAGE. MDCK I cells were treated with inhibitors for 60 minutes, and p100/p120 analysed by immunoblot. Staurosporine (100 nM), KT5926 (1 μM), Gö 6926 (1 μM) and ML-7 (100 μM) all increased the electrophoretic mobility of p100 and p120, consistent with dephosphorylation. Gö 7874 (1 μM), KN-62 (100 μM), H7 (300 μM), Bisindolylmaleimide I (2.5 μM) and Ro 31-8425 (5 μM) had no effect on p100/p120 mobility.
KN-62, a CaM kinase II inhibitor (Tokumitsu et al., 1990), had no effect on p100/p120. Gö 6976 was able to alter p100 and p120 electrophoretic mobility, but the structurally related compound Gö 7874 had no effect. Gö 6976 and Gö 7874 were originally identified as inhibitors of specific PKC isoforms (Qatsha et al., 1993; Martiny-Baron et al., 1993; Kleinschroth et al., 1995). H-7, an inhibitor of a range of kinases including MLCK and PKC (Hidaka et al., 1984; Kawamoto and Hidaka, 1984), was not capable of inducing p100/p120 dephosphorylation, and neither of the PKC inhibitors bisindolylmaleimide I and Ro 31-8425 affected p100/p120 phosphorylation. Thus there is a distinctive pattern of compounds that can induce a p100/p120 mobility shift. It is probable that the p100/p120 kinase is inhibited by Gö 6976, KT5926, staurosporine and ML-7, but is insensitive to Gö 7874, Ro 31-8425, bisindolylmaleimide I and KN-62.

4.5 Identification of potential GSK-3β phosphorylation sites in p100/p120

A potential mechanism for p100/p120 dephosphorylation is via the PKC-induced inhibition of GSK-3β. GSK-3β has been shown to phosphorylate β-catenin, which, like p120, is a member of the Armadillo family of proteins. This is a vital component of the Wnt/Wingless signalling pathway. PKC can phosphorylate and inhibit GSK-3β in vitro, and has been implicated in the Wingless-induced inactivation of GSK-3β. If p100/p120 are substrates for GSK-3β, PKC could promote dephosphorylation of these proteins by inhibiting GSK-3β. Although a number of substrates for GSK-3β have been identified, there is no strict consensus GSK-3β phosphorylation site. Generally, a motif consisting of SxxxS, where X is any amino acid, becomes phosphorylated (Plyte et al., 1992). In a number of GSK-3β substrates, such as glycogen synthase and inhibitor-2, the substrate must be primed by prior phosphorylation of the C-terminal serine residue of the SxxxS motif in order for the more N-terminal serine residue to be phosphorylated by GSK-3β. There are eight
SxxxS motifs in the p120 sequence (fig. 4.5), so p100 and p120 may be potential substrates for GSK-3β. However, the poorly defined nature of the GSK-3β recognition sequence makes it very difficult to predict accurately which proteins might be substrates. The SxxxS motif is relatively unspecific, and so is likely to occur in many proteins. The presence of SxxxS motifs in a protein does not necessarily prove that it is a substrate for GSK-3β.

4.6 GSK-3β protein expression and activity in MDCK I cells

Whole cells lysates of MDCK I cells were separated by SDS-PAGE, transferred to nitrocellulose, and analysed by western blot. GSK-3β antibodies recognised a single band at 46 kD, the expected molecular weight of this protein (fig. 4.6 A). It was also possible to immunoprecipitate this protein from MDCK I cells (fig. 4.6 B); immunoblotting of these GSK-3β immunoprecipitates again showed a single band at approximately 46 kD. Immunoprecipitated GSK-3β could also be assayed by its ability to phosphorylate a phospho-GS peptide. The sequence of this peptide, which is derived from glycogen synthase, is; YRRAAVPPS.PSLS.RHSS.PHQpSEDEEE. The phosphorylation of serine 21 in the sequence is necessary for the peptide to be a GSK-3β substrate (Van Lint et al., 1993). The sites phosphorylated by GSK-3β are underlined. A similar peptide with this serine substituted with alanine ([Ala21]GS peptide) is not a substrate for GSK-3β. GSK-3β purified by immunoprecipitation from MDCK I cells was capable of phosphorylating the phospho-GS peptide (fig. 4.7 A). The [Ala21]GS peptide was not phosphorylated under these conditions. Immunoprecipitated Cdc2 kinase or focal adhesion kinase (FAK) were unable to phosphorylate the phospho-GS peptide. Thus, measurement of the incorporation of 32P into phospho-GS peptide proved a specific assay for the activity of GSK-3β.

PKC has been shown to inactivate GSK-3β in vitro. Stimulation of cells with EGF can also inactivate GSK-3β (Saito et al., 1994; Eldar-Finkelman et al., 1995). To see if GSK-3β was inactivated in MDCK I cells in response to phorbol ester, GSK-3β
Residue number

1  MDDSEVESTASILASVKEQAQFEKLTRALEEEERRHVSAQ
41 LERVRVSQPQANSLMANGTTLTRRRHGNRFVGDADLERQKF
81 SDLKLNPGQDHNLLLYSTIPRMQEPGQIVETYTEDPEGA
121 MSVSVSTETTDDGTTRRTETTVKKVKTMTRTVQPVPMGP
161 DGLPVDASAVSNNYIQTGLRDFRKNNGPGPGPYVGQAGTA
201 TLPRNFHYPPDGYGRHEDGYPGGSDNYGSLSRVRTIEER
241 YRPSMEGYPASRDQGYGPQPQVRVGGSSVDLHHRFHPepy
281 GLeddqRSMGYDDLGYGMMSDYGTRARRGTPSDPRRRLRS
321 YEDMIGEVEVPPDQYWWAFOAPHERGSLASLDLSLKGMPPP
361 SNWRQPELPEVIAMLGFRLDAVKSNAAAYLQHLCYRNDKV
401 KTDVAKLKGIPILVGLDLLPKEVHLCAGALKNISGRD
441 QDNKIAIKNCDGVPALVRLRKRMDLMTEVITGTTLWNLS
481 SHDSIKMEIVDHALHALTDEVIIIPHSGWREPNEDECKPRH
521 IWESEVLNTAGCLRNVESTERSERRKLERCDGLVDALIF
561 UVQAEIIGKDSDSLKVENCVCCLLNRNSQVHREIPQARY
601 QEALPTVANSTGPHAASCFGAKKKGKPTEDPADTVDF
641 PKRTSPARGYELLFQPEVVRITYISLLKESNTPAILEASAG
681 AIQNLCAGRWTRYIRSANLREQEKALASARELLTSEHERV
721 VKAASGARLNLVADARKELIGKHARPNLWKNLPGQQNS
761 SNWFSEDIVSVILNNTINEVIAENLEAAKLRTEQQIEKLV
801 LNKSSGNRSEKEVRAAALVQTIWGYKBRLKPELEKEGWKK
841 SDFQVNLNNASRSCSHVDDSITLPIDRNQKSDNNYSTL
881 NERGDNRLRTLDRESGDLGDMEFPLKGAFLMQKI

Fig 4.5. p120 amino acid sequence (murine).

p120 protein contain 911 amino acid residues. The GSK-3β motifs (SxxxS) are highlighted, and the potential sites of GSK-3β phosphorylation are shown in red. The serine residue that occurs four amino acids upstream of the GSK-3β target serine must be phosphorylated in order for GSK-3β to phosphorylate its target residue.
Fig. 4.6. GSK-3β in MDCK I cells

GSK-3β can be detected in MDCK I cells by western blotting (A). A single protein of 46 kD is recognised by the antibody, with a 1:500 dilution of primary antibody giving the strongest signal. (B) GSK-3β can be purified from MDCK I cells by immunoprecipitation. Approximately $10^6$ MDCK I cells were lysed into TDS lysis buffer, and GSK-3β immunoprecipitated using increasing quantities of primary antibody as indicated. Proteins were separated by SDS-PAGE, and immunoblotted with anti-GSK-3β antibody. An anti-Ras mouse monoclonal antibody was used as a control.
Fig. 4.7. GSK-3β peptide substrate phosphorylation

(A) Focal adhesion kinase (FAK), Cdc 2 and GSK-3β were immunoprecipitated from MDCK I cells under TDS lysis conditions. The purified proteins were used in a kinase assay in the presence of [³²P]ATP. Incorporation of ³²P into a GSK-3β peptide substrate (phospho-GS peptide), or a control peptide, which is not a substrate for GSK-3β ([Ala₂¹]GS peptide), was assayed by scintillation counting of peptide bound to P81 chromatography paper. Only GSK-3β caused phosphorylation of the phospho-GS substrate peptide, showing this assay specifically measures GSK-3β activity.

(B) MDCK I cells were treated with 200 nM PDB for 30 minutes, 50 nM EGF for 15 minutes or DMSO (1:1000) for 30 minutes as a control. Cells were then lysed into TDS lysis buffer, and cellular GSK-3β extracted by immunoprecipitation. The purified GSK-3β was then assayed for its ability to phosphorylate the phospho-GS peptide substrate.
was immunoprecipitated from PDB-treated MDCK cells, and enzyme activity assayed by phosphorylation of phospho-GS peptide. Neither PDB or EGF caused a significant decrease in the activity of GSK-3β (fig 4.7 B). The biological activity of PDB and EGF in this assay was demonstrated by the effect of PDB on monolayer resistance, and the ability of EGF to induce tyrosine phosphorylation of the EGF receptor (data not shown). It may be that EGF and PKC do not promote inhibition of GSK-3β in MDCK I cells. However, this approach assumes that any inhibitory effect of PDB or EGF on GSK-3β, be it phosphorylation or allosteric modification, is preserved during the immunoprecipitation of the GSK-3β enzyme. It is possible that this is not the case.

4.7 p100/p120 is not a substrate for GSK-3β in vitro

In order to see if p100 and p120 can act as substrates for GSK-3β in vitro, MDCK I cells were treated with or without PDB, and the p100/p120 were isolated by immunoprecipitation. Thus both normally phosphorylated and dephosphorylated p100/p120 could be used as substrate for GSK-3β. β-catenin was also purified from these cells as a positive control, since β-catenin is known to be a GSK-3β substrate. The immunoprecipitated p100/p120 or β-catenin were mixed with recombinant GSK-3β in buffer supporting kinase activity and [γ-32P]-ATP. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose, and phosphate incorporation analysed by autoradiography. β-catenin became phosphorylated, but p100 and p120 did not (fig. 4.8). Thus p100 and p120 are not substrates for GSK-3β in vitro. This argues against GSK-3β being the p100/p120 kinase in vivo, although it could be argued that additional co-factors are required for in vivo phosphorylation.

4.8 Inhibition of GSK-3β with kinase inhibitors

p100/p120 dephosphorylation can be induced by certain kinase inhibitors (see fig. 4.4). If GSK-3β is the p100/p120 kinase, it should be inhibited by these inhibitors. The effect of the kinase inhibitors staurosporine, KT5926, Gö 6976,
Fig. 4.8. p100 and p120 are not substrates for GSK-3β in vitro.

10^6 MDCK I cells were treated for 30 minutes with 200 nM PDB (+), or DMSO control (-), and the p100/p120 purified by immunoprecipitation in TDS lysis buffer. Both phosphorylated and hypophosphorylated p100/p120 were then incubated with recombinant GSK-3β in the presence of [γ-32P]-ATP. β-catenin was also immunoprecipitated from these cells, and used in the kinase assay as a positive control, since β-catenin is known to be a substrate for GSK-3β. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose, and phosphate incorporation analysed by autoradiography. β-catenin became phosphorylated, but p100 and p120 did not. β-catenin was not phosphorylated in the absence of GSK-3β enzyme (data not shown). Thus p100 and p120 are not substrates for GSK-3β in vitro. Panel A and B show two separate experiments; in (A) 50 mUnits of GSK-3β were used per reaction; in (B) 100 mUnits GSK-3β were added.
bisindolylmaleimide I and Ro 31-8425 on the ability of recombinant GSK-3β to phosphorylate the phospho-GS peptide was assayed. Staurosporine and Gö 6976 were able to inhibit GSK-3β, but KT5926 did not (fig 4.9). However, the PKC inhibitors bisindolylmaleimide I and Ro 31-8425 (which do not inhibit the p120 kinase) were potent inhibitors of GSK-3β. Similar results were obtained if GSK-3β purified from MDCK I cells was used in the phosphorylation assay (data not shown). There may be more than one p100/p120 kinase, perhaps with different sensitivity to kinase inhibitors. The inability of KT5926 to inhibit GSK-3β does not preclude the possibility that GSK-3β is a p100/p120 kinase; it is possible that GSK-3β is the Gö 6976/staurosporine sensitive p100/p120 kinase, and some other kinase is inhibited by KT5926, and this leads to p100/p120 dephosphorylation in response to this inhibitor. However, the inhibition of GSK-3β by bisindolylmaleimide I and Ro-31-8425 is inconsistent with this enzyme being a p100/p120 kinase, since neither bisindolylmaleimide I or Ro 31-8425 promote p100/p120 dephosphorylation in vivo; indeed they function to block the PDB-induced p100/p120 mobility shift (see fig. 3.6).

4.9 The MAP kinase pathway is not the pathway by which PKC induces p100/p120 dephosphorylation

Another pathway activated by PKC is the MAP kinase pathway. I investigated whether this pathway was activated in MDCK I cells in response to addition of PDB, and whether inhibition of this pathway blocked the p100/p120 dephosphorylation. MAP kinases are activated by phosphorylation on threonine and tyrosine residues (Anderson et al., 1990). These phosphorylation events cause a mobility shift of the p42 MAP kinase, Erk-2, seen in western blots. Also, the tyrosine phosphorylation of the p42 and p44 MAP kinases, Erk-2 and Erk-1, at position Tyr204 (Payne et al., 1991) can be detected by a commercially available phospho-Erk antibody which recognises the tyrosine phosphorylated (and thus activated) forms of Erk-1 and Erk-2.
Fig. 4.9 Gsk-3β inhibition by various kinase inhibitors.
The activity of recombinant GSK-3β was assayed, by measuring the incorporation of 
$[^{32}P]$phosphate into a phospho-GS peptide substrate. 25 mUnits of enzyme were 
incubated in kinase assay buffer with 5 μg of substrate and inhibitors at the designated 
concentration for 10 minutes. [γ-32P]ATP was then added, the reaction incubated at 
30°C for 10 minutes, and the phospho-GS peptide extracted by binding to P81 
chromatography paper. The counts incorporated into the peptide provided a measure of 
the activity of the enzyme. Each column on the graph shows the average of two 
experiments, with standard deviation represented by error bars. Abbreviations used: 
KT, KT5926; St, staurosporine; Go, Gö 6976; Bis, bisindolylmaleimide I; Ro, Ro 31-
8425. Similar results were obtained in three separate experiments, and also in 
experiments using GSK-3β purified from MDCK I cells instead of recombinant GSK-
3β (data not shown).
Addition of PDB or treatment of MDCK I cells with EGF promoted activation of the MAP kinase pathway, as judged by phosphorylation of Erk-1 and Erk-2 (fig. 4.10 B, C). However, EGF did not induce dephosphorylation of p100 and p120 (fig. 4.10 A). The phosphorylation and activation of these MAP kinases could inhibited by the MAPK kinase inhibitor PD 98059 (Dudley et al., 1995). The inhibition of the MAP kinase pathway was not quite complete, as there was still some increase in phosphorylation of Erk-1 and Erk-2 in response to PDB and EGF, even in the presence of PD 98059. However, densitometric scanning showed that PD 98059 reduced the level of phosphorylation of Erk-1 and Erk-2 in PDB-treated cells to less than that seen in normal untreated cells. The level of Erk-1 and Erk-2 phosphorylation in response to PDB was reduced by 60% in the presence of PD 98059, but this inhibitor had no effect on p100/p120 dephosphorylation. Since EGF activated the MAP kinase pathway but did not promote p100/p120 dephosphorylation, and reducing the levels of activated MAP kinases to less that in resting cells has no effect on PDB-induced dephosphorylation of p100/p120, it is unlikely that the MAP kinase pathway is involved in the dephosphorylation of p100 and p120.

4.10 BAPTA does not induce p100/p120 dephosphorylation

Addition of the Ca\(^{2+}\)-chelator BAPTA to epithelial cells dissociates intercellular adhesion, by disrupting cadherin-cadherin interaction. Addition of 4 mM BAPTA caused a disruption of tight junctions within 5 minutes, as assayed by resistance across an MDCK I monolayer. Analysis of p100/p120 mobility by western blot demonstrated that addition of BAPTA did not lead to dephosphorylation of these proteins (fig. 4.11). Thus, the p100/p120 does not occur simply as a consequence of disruption of the adherens junction. Also, the disruption of adherens junctions that occurs in response to BAPTA did not inhibit the ability of PDB to induce p100/p120 dephosphorylation (fig. 4.11). Therefore dephosphorylation of p100/p120 is not dependent on intact cadherin-mediated cell-cell adhesion.
Fig. 4.10  p100/p120 dephosphorylation and the MAP kinase pathway.
MDCK I cell were treated with DMSO or the MAP kinase inhibitor PD 98059 (50 μM) for 15 minutes. DMSO, 200 nM PDB or 50 nM EGF was then added for a further 15 minutes, cells were lysed into Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Activation of the MAP kinase pathway can be assayed by a band shift of the 42 kD MAP kinase (Erk-2), due to its phosphorylation on Tyr204 (A), or by use of an antibody which recognises phosphorylated (and thus activated) MAP kinases, Erk-1, and Erk-2 (B). These assays show that the MAP kinase pathway is activated by both PDB and EGF in MDCK I cells. This pathway is blocked by PD 98059. This block is not quite complete, as there is a slight increase in phosphorylation of Erks in cells treated with PDB, even in the presence of PD 98059, as judged by phospho-Erk blotting (B). However, the level of phosphorylated Erk-1 and Erk-2 in PDB-treated cells in the presence of PD 98059 is similar to that in normal untreated cells, and is much lower than in PDB-treated cells. Panel (C) shows a β-catenin re-probe of the phospho-Erk blot shown in panel (B) to demonstrate equal protein loading. PDB induced p100/p120 dephosphorylation, even in the presence of PD 98059, whereas EGF did not promote a p100/p120 mobility shift (D).
Fig. 4.11 BAPTA and p100/p120 electrophoretic mobility.
Treatment of MDCK I cells with 4 mM BAPTA for 15 minutes had no effect on the electrophoretic mobility of p100/p120. Pretreatment of cell with BAPTA did not block the PDB-induced dephosphorylation of p100/p120.
4.11 Discussion

The experiments described in this chapter attempted to identify the players involved in p100/p120 dephosphorylation in response to PDB. These players remain, at present, unknown, but a number of possibilities have been excluded. A variety of data suggest that GSK-3β is not a p100/p120 kinase; GSK-3β did not appear to be inhibited by PDB stimulation of MDCK I cells; p100/p120 are not substrates for GSK-3β \textit{in vitro}; GSK-3β is effectively inhibited by bisindolylmaleimide I and Ro 31-8425, but these inhibitors have no effect of p100/p120, and so do not inhibit the p100/p120 kinase(s).

MAP kinases do not appear to be involved in the pathway by which PKC promotes p100/p120 dephosphorylation. Both PDB and EGF activated the MAP kinase pathway in MDCK I cells, but EGF did not promote p100/p120 dephosphorylation. Inhibition of the MAP kinase pathway by PD 98059 had no effect on the dephosphorylation of p100/p120 in response to PDB. However, since there was a slight activation of Erk-1 and Erk-2 in response to PDB, even in the presence of PD 98059, this pathway cannot be categorically ruled out.

The pathway by which PKC promotes p100/p120 dephosphorylation does not require intact cell-cell adhesions, since disruption of cadherin-mediated cell adhesion by chelation of extracellular Ca$^{2+}$ with BAPTA did not prevent the PKC-induced p100/p120 dephosphorylation.

Although the phosphopeptide mapping approach proved to be unsuccessful in identifying the sites of dephosphorylation in p100/p120, the existence of a 70 kD breakdown product of p100/p120 was demonstrated during the immunoprecipitation of phosphate-labelled p100/p120. This protein is recognised by the pp120 antibody, and so contains the C-terminal region of the p120 protein. It is also subject to dephosphorylation in response to PDB, and so it is likely that the sites of serine/threonine dephosphorylation occur in the C-terminal 70 kD region of p100 and p120.
5. Investigating the physiological relevance of p100/p120 dephosphorylation

5.1 Introduction

In chapter 3, I showed that the adherens junctions proteins p100 and p120 are dephosphorylated in response to PDB in epithelial cells. It is possible that p100/p120 dephosphorylation is a necessary step in the disruption of intercellular adhesion that occurs when PKC becomes activated. Perhaps PKC leads to dephosphorylation of p100 and p120, which in turn causes a functional disruption of the adherens junctions. Cadherin-mediated cell-cell adhesion also occurs in endothelial cells, and in some fibroblasts (Yonemura et al., 1995). In endothelia, activation of PKC can lead to disruption of intercellular adhesion. I therefore investigated whether activation of PKC promotes p100/p120 dephosphorylation in endothelia and fibroblasts.

Since tight junction integrity is dependent on intact adherens junctions, a signalling pathway that affected the adherens junction could provide a mechanism for opening the tight junction. In MDCK I cells, p100/p120 dephosphorylation in response to PDB occurs with similar kinetics to the drop in resistance (see fig. 3.7). The p100/p120 dephosphorylation in response to PDB can be reversed by addition of bisindolylmaleimide, and this correlates with recovery of the original monolayer resistance. I investigated whether p100/p120 dephosphorylation correlated with disruption of tight junctions in other epithelial cells, and whether the p100/p120 dephosphorylation that occurs in response to kinase inhibitors correlated with disruption of tight junctions. The effect of dephosphorylation on the cellular distribution and stability of p100/p120 was also examined.

5.2 p100/p120 dephosphorylation in endothelial cells

Endothelial cells have adherens junctions, and in many instances, PKC activation has been shown to cause an increase in endothelial paracellular permeability.
PKC caused an increase in p100 and p120 electrophoretic mobility in a wide range of endothelial cell lines, including primary pig brain endothelial cells (fig. 5.1). Of all the endothelial cell lines tested, only in rat brain endothelial (RBE4) cells was p100/p120 unaffected by PDB treatment. Phosphate labelling of p100/p120 from EA.hy926 cells showed that this mobility shift was due to dephosphorylation (fig. 5.2).

5.3 p100/p120 dephosphorylation in fibroblasts

Some fibroblasts have been shown to express p120 isoforms, and cell-cell adhesion in fibroblasts can be mediated through cadherin-cadherin interactions. Therefore, the mobility of p120 isoforms in NIH 3T3 fibroblasts was investigated. Addition of PDB caused a clear alteration of p100 and 120 mobility (fig. 5.3 A). Phosphate labelling analysis demonstrated that this altered mobility was indeed due to dephosphorylation of the p120 isoforms (5.3 B, C). Thus the dephosphorylation of p120 isoforms in response to activation of PKC appears to be a widespread phenomenon, occurring in many endothelial cells, and even in fibroblasts.

5.4 PDB effects on tight junctions in Caco-2, MDCK II and LLC-PK1 cells

p100 and p120 dephosphorylation occurs in response to PDB stimulation of various epithelial cell lines, including Caco-2, LLC-PK1, and MDCK II cells (see fig. 3.10). The resistance across cell monolayers plated on porous filters was measured in response to stimulation of PKC with PDB (fig. 5.4). LLC-PK1 and Caco-2 cells both show a drop in resistance in response to PDB, in line with previously published data (Soler et al., 1993; Stenson et al., 1993), but the resistance across MDCK II cell layers is not significantly decreased. However, analysis of [14C]sucrose flux showed that paracellular transport of this tracer increased in response to PDB in these all these cells. Thus the p100/p120 dephosphorylation correlates with opening up of intercellular junctions in three different epithelial cell lines.
Fig. 5.1. $p_{100}/p_{120}$ band shift in endothelial cells.
A number of endothelial cell lines were treated with either DMSO (-) or 200 nM PDB (+) for 30 minutes, lysed into sample buffer and separated by SDS-PAGE. Proteins were transferred to nitrocellulose and blotted with antibody recognising $p_{100}/p_{120}$. The positions of $p_{100}$ (▲) and $p_{120}$ (▲) are indicated. Cell lines used were; PBEC, primary pig brain endothelial cells; BAEC, bovine aortic endothelial cells; EA.hy926 and ECV 304, HUVEC derived-endothelial cells; HUVEC, human umbilical vein endothelial cells; RBE4, rat brain endothelial cells. Only in RBE4 cells were $p_{100}$ and $p_{120}$ unaffected by PDB treatment.
Fig. 5.2 Dephosphorylation of p100/p120 in EA.hy926 endothelial cells.

(A) Following overnight labelling with $^{32}$P orthophosphate, EA.hy926 cells were exposed for 30 minutes to 200 nM PDB, or vehicle DMSO only, and lysed in TDS buffer. p100 and p120 were immunoprecipitated, blotted and labelled protein was visualised by autoradiography. The level of phosphate in p100 and p120 from control cells, relative to that from PDB-treated cells, was determined by densitometric scanning. The same filters were then probed with p100/p120 antibody and the level of protein was determined by densitometry. The amount of phosphate per unit protein could be calculated. The results obtained from scanning p120 and p100 signals from the autoradiographs are shown in panel B and C respectively. $^{32}$P and protein signal of p100 and p120 are measured in OD.mm.mm. 'Pn' indicates 'phosphate level normalised for protein'. Phosphate content of p100 and p120 was reduced by 14 ± 1%, and 15 ± 2% respectively (average of three experiments).
A

<table>
<thead>
<tr>
<th></th>
<th>Set 1</th>
<th>Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td>PDB</td>
</tr>
<tr>
<td>32P-signal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

**p120 phosphorylation scanning data**

<table>
<thead>
<tr>
<th>PDB Treatment</th>
<th>Set 1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>32P (OD.mm.mm)</td>
<td>33.2</td>
<td>30.5</td>
<td>33.0</td>
</tr>
<tr>
<td>Protein (OD.mm.mm)</td>
<td>17.8</td>
<td>19.6</td>
<td>23.1</td>
</tr>
<tr>
<td>32P/protein (Pn)</td>
<td>1.87</td>
<td>1.55</td>
<td>1.43</td>
</tr>
<tr>
<td>Pn (Control) : Pn (PDB)</td>
<td>1 : 0.83</td>
<td>1 : 0.86</td>
<td></td>
</tr>
</tbody>
</table>

C

**p100 phosphorylation scanning data**

<table>
<thead>
<tr>
<th>PDB Treatment</th>
<th>Set 1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>32P (OD.mm.mm)</td>
<td>30.0</td>
<td>26.9</td>
<td>27.9</td>
</tr>
<tr>
<td>Protein (OD.mm.mm)</td>
<td>16.3</td>
<td>16.8</td>
<td>20.9</td>
</tr>
<tr>
<td>32P/protein (Pn)</td>
<td>1.84</td>
<td>1.60</td>
<td>1.33</td>
</tr>
<tr>
<td>Pn (Control) : Pn (PDB)</td>
<td>1 : 0.87</td>
<td>1 : 0.86</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 5.3 Dephosphorylation of p120 in NIH 3T3 fibroblasts.

(A) Immunoblotting of NIH 3T3 lysates demonstrated that p100 and p120 show altered electrophoretic mobility in response to PDB (at 200 nM for 30 minutes). (B): Following overnight labelling with $[^{32}P]$orthophosphate, NIH 3T3 cells were exposed for 30 minutes to 200 nM PDB, or vehicle only, and lysed in TDS buffer. p100 and p120 were immunoprecipitated, blotted and labelled protein was visualised by autoradiography. p120 is the predominant isoform expressed in NIH 3T3 cells, and the level of phosphate in p120 from control cells, relative to that from PDB-treated cells, was determined by densitometric scanning. To normalise for slight differences in protein loading, the same filters were then probed with p120 antibody, and the level of p120 protein was determined by densitometry. Thus, the amount of phosphate per unit protein could be calculated. Panel C shows the results of the scanning of the autoradiographs shown in panel B, with $^{32}P$ and protein signal of the p120 protein measured in OD.mm.mm. $P_n$ indicates 'phosphate level normalised for protein'.
Fig. 5.4 Resistance and sucrose flux across different epithelia in response to activation of PKC

Paracellular resistance and flux was measured across Caco-2 (A), LLC-PK₁ (B), and MDCK II (C) cell monolayers. All showed an increase in [¹⁴C]sucrose flux in response to PDB. The levels of [¹⁴C]sucrose flux could only be explained by increased paracellular traffic, since levels of transcytosis across MDCK cells are several orders of magnitude lower than the values we obtained (von Bonsdorff et al., 1985). In Caco-2 and LLC-PK₁ cells there was a concomitant drop in resistance. The resistance across MDCK II monolayers was not significantly altered in response to PDB. Starting resistance values were: Caco-2, 326.5±16.2 Ω.cm²; LLC-PK₁, 117±6.1 Ω.cm²; MDCK II, 140.4±2.8 Ω.cm². Each point represents the mean of three separate filters, standard deviation indicated by error bars.
Resistance sucrose flux

A

% Original resistance

Time (min)

120
100
80
60
40
20
0

B

% Original resistance

Time (min)

120
100
80
60
40
20
0

C

% Original resistance

Time (min)

120
100
80
60
40
20
0

DMSO

PDB

Time (hours)
5.5 Effect of kinase inhibitors on paracellular transport

The kinase inhibitors Gö 6976, KT5926 and staurosporine all promote p100/p120 dephosphorylation. The effect of KT5926 and Gö 6976 on resistance and sucrose flux across MDCK I cell monolayers was investigated (fig. 5.5). Both inhibitors caused a decrease in resistance, but there was no increase in flux; if anything there was a slight decrease in sucrose flux in response to these inhibitors. Since KT5926 and Gö 6976 do not increase paracellular flux, the effect on resistance is unlikely to be due to disruption of tight junctions. Preliminary experiments showed that the resistance drop across MDCK I cell layers in response to KT5926 could be almost completely blocked if medium was used in which Cl⁻ ions had been replaced with the plasma membrane-impermeable analogue gluconate (data not shown). This suggests that the resistance drop in response to KT5926 is due to an effect on Cl⁻ channels, rather than intercellular junctions. Therefore dephosphorylation of p100/p120 alone is not sufficient to promote a disruption of intercellular junctions. However, it is still possible that p100/p120 dephosphorylation is a necessary step in the disruption of adhesion between cells in response to PKC activation.

5.6 E-cadherin-associated p100/p120 is dephosphorylated in response to PDB

It has been shown that although some of the p100/p120 in cells is associated with adherens junctions, there is also a large pool that is not complexed with cadherins, at least by immunoprecipitation analysis of Triton X100 solubilised proteins (see for example Reynolds et al., 1994). In order to determine which of these p100/p120 pools are subject to dephosphorylation, MDCK I cells were treated with PDB, extracted using Triton lysis buffer, which preserves protein-protein interactions, and immunoprecipitated with antibodies recognising E-cadherin. This procedure serves to isolate only that portion of the cellular p100/p120 that associates with cadherin. Following SDS-PAGE and transfer to nitrocellulose, the p100 and p120 present in
Fig. 5.5. Resistance and paracellular flux across MDCK I monolayers in response to the kinase inhibitors KT5926 and Gö 6976

(A) MDCK I cell monolayers were treated with KT5926 (1 μM), Gö 6976 (1 μM) or DMSO (1:1000) as a control. Resistance was measured at the times indicated.

(B) 14C-sucrose flux across MDCK I monolayers in response to KT5926 (1 μM), Gö 6976 (1 μM) or DMSO. Both resistance and flux data points represent the mean of three monolayers, with standard deviation indicated by error bars.
E-cadherin immunoprecipitates were detected by immunoblotting (fig. 5.6 A). It can be seen that the E-cadherin-associated p100/p120 is dephosphorylated in response to PDB. Similarly, where antibodies against β-catenin were used to immunoprecipitate the adherens junction complex, the p100/p120 attached to the complex was shown to be subject to dephosphorylation in response to PDB. Immunoblot analysis of the non-E-cadherin-associated p100/p120 pool revealed that this too was subject to dephosphorylation (data not shown). Even prolonged exposure to PDB did not cause an alteration in the association of E-cadherin with p120 (fig. 5.6 B, C). [35S]methionine labelling followed by immunoprecipitation of p100/p120 under conditions that preserved protein-protein interactions confirmed that there was no change in the association of p100 and p120 with E-cadherin, α-catenin or β-catenin (fig. 5.6 D).

5.7 Immunofluorescence studies of adherens junctions in response to PDB

Immunofluorescence studies in MDCK I cells revealed no apparent change in the localisation of p100 and p120 after PDB treatment. Similarly, there was no change in localisation of E-cadherin or β-catenin (fig. 5.7). Thus, activation of PKC does not promote gross disassembly of adherens junctions.

5.8 p100/p120 stability in MDCK I cells

The stability of p100 and p120 following PDB-induced dephosphorylation was investigated by pulse-chase analysis. MDCK I cells were metabolically labelled for 48 hours with [35S]methionine. The radioactive methionine was then washed away, and the cell incubated in normal media containing either PDB, or DMSO as a control. Cells were sampled after 2, 4 and 8 hours, and p100 and p120 purified by immunoprecipitation, and separated by SDS-PAGE. Incorporation of [35S]methionine was analysed by autoradiography (fig 5.8 A). There was no significant difference in
Fig. 5.6  p100 and p120 remain associated with the adherens junction complex following PDB stimulation

MDCK I cells treated for 30 minutes with either 200 nM PDB or vehicle were extracted into Triton lysis buffer, and the adherens junction complex immunoprecipitated (IP) with antibodies recognising E-cadherin, or β-catenin. Following SDS-PAGE, and transfer to nitrocellulose, the immunoprecipitates were probed with antibodies to p100/p120 (A). E-cadherin-associated p100 and p120 were dephosphorylated in response to PDB. Similarly, p100/p120 associated with β-catenin was subject to dephosphorylation. Prolonged activation of PKC in MDCK I cells had no effect on the association between E-cadherin and p100/p120. E-cadherin immunoprecipitates probed with p100/p120 antibody (B), or p100/p120 immunoprecipitates probed with E-cadherin antibody (C) both demonstrated that E-cadherin-p100/p120 interaction was maintained even after 4 hours exposure to PDB. Immunoprecipitation of p100 and p120 from [35S]methionine-labelled cells, under conditions where protein-protein interactions were maintained, showed that the association of p100 and p120 with other components of the adherens junction was unchanged following PDB treatment (D). Location of E-cadherin, α-catenin and β-catenin are indicated. The presence of p100, seen as a diffuse band in untreated cells, is indicated. On addition of PDB, p100 co-migrates with β-catenin, so this diffuse band seems to disappear.
A

E-Cadherin IP
- + - +

β-catenin IP
- +

p120
p100

B

Time of exposure to 200 nM PDB (minutes)

0 15 30 60 120 240

E-cadherin IP

p120
p100

probe

C

p120 IP

E-cadherin

probe

D

IP: p100/p120
(35S-methionine labelled)

0 30 120 minutes

200

kD

p100

E-cadherin

α-catenin

β-catenin
Fig. 5.7 Location of adherens junction proteins in untreated MDCK I cells and MDCK I cells stimulated with PDB.

MDCK I cells grown on 13 mm glass coverslips were treated with 200 nM PDB or DMSO for 30 minutes, fixed in paraformaldehyde and immunolabelled. The distribution of E-cadherin (E-cad), β-catenin (β-cat) and p100/p120 (p120) as seen with a x40 objective are indicated. In addition, distribution of p100/p120 as seen with a x100 objective is shown. The scale bar represents 40 μm for the x40 objective, 10 μm for the x100 objective. There was no alteration in cellular distribution of any of these proteins in response to PDB.
Fig. 5.8 Stability of p100/p120 is unchanged after dephosphorylation

MDCK I cells were labelled metabolically with $[^{35}S]$methionine for 48 hours. The cells were then washed thoroughly in non-radioactive medium, and treated with DMSO or 200 nM PDB, and sampled at the times indicated. p100 and p120 were purified by immunoprecipitation in TDS lysis buffer, separated by SDS-PAGE, and $[^{35}S]$methionine incorporation measured by autoradiography. Panel A shows an experiment where p100/p120 protein was sampled at 2, 4 and 8 hours after chasing away the $[^{35}S]$methionine. Panel B shows a separate experiment, in which the stability of p100/p120 was tested after 3, 6, 9 and 12 hours stimulation with PDB. In both cases, there was no significant difference in the stability of dephosphorylated p100/p120 compared with the normally phosphorylated forms. There is very little turnover of p100/p120 over 8-12 hours either in control cells, or PDB-treated cells, which is consistent with the long half-life of p100/p120 in MDCK cells (Staddon et al., 1995a).
the stability of phosphorylated versus dephosphorylated p120. A second experiment, analysing \([^{35}\text{S}]\text{methionine incorporation at 3, 6, 9 and 12 hours confirmed that there is no change in p100/120 stability in MDCK I cells following PDB stimulation (fig. 5.8 B)}.

5.9 Discussion

p100 and p120 dephosphorylation occurs in response to PDB in endothelia and fibroblasts as well as epithelia. Therefore this signalling pathway is not limited to specific cells, but is a common feature of many different cell types. Epithelia, endothelia and fibroblasts all show cadherin-mediated cell-cell adhesion, and in all these cells, p100 and p120 are associated with cadherins, and potentially regulate their function. The cadherin-associated pool of p100 and p120 in epithelial cells is subject to dephosphorylation, which demonstrates that this PKC-mediated signalling pathway acts directly on the adherens junction. Since p100/p120 dephosphorylation in response to PDB correlates with disruption of intercellular junctions in MDCK I, MDCK II, LLC-PK1 and Caco-2 epithelial cells, there is a possibility that p100/p120 dephosphorylation plays a key role in the pathway by which PKC affects epithelial tight junctions.

The data obtained using the MDCK strain II cells represent an anomalous situation. PDB treatment clearly caused dephosphorylation of p100 and p120 in these cells. However, even though transcellular electrical resistance across these monolayers was not greatly reduced by PDB, there was an increase in paracellular sucrose flux. The tight junction protein occludin has recently been overexpressed in MDCK cells (Balda et al., 1996; McCarthy et al., 1996) leading to an increase in transcellular electrical resistance with, unexpectedly, a concomitant increase in tracer flux. Tight junctions can be viewed as a series of one or more diffusion barriers. It is possible that solute permeation, a gradual process, may be achieved step-wise by the asynchronous opening of individual barriers within the tight junction complex. Electrical resistance
represents an instantaneous read-out of the ionic permeability of the serial diffusion barriers within the tight junction complex, and so would require a synchronous increase in permeability. The effect of PDB on the MDCK II cells may represent a situation where asynchronous opening of diffusion barriers to solute occurs, allowing, over time, increased tracer flux, but does not simultaneously occur in individual diffusion barriers, therefore not yielding a decrease in transcellular electrical resistance.

If the phosphorylation state of p100/p120 alone is a key regulator of permeability it would be expected that selective manipulation of phosphorylation should give predictable changes in permeability. Where p100/p120 dephosphorylation was induced by the kinase inhibitors Gö 6976 and KT5926 (fig. 5.5), this was not associated with increased paracellular sucrose flux. It is possible that p100/p120 dephosphorylation could be involved in permeability regulation but additional effects of the inhibitors on other kinases prevent the physiological response. Alternatively, p100/p120 dephosphorylation may not be sufficient to trigger a permeability increase. It is likely that PKC activation acts to increase tight junction permeability by concerted regulation of a number of events, including p100/p120 dephosphorylation.

If p100/p120 phosphorylation affects paracellular permeability, it might be expected that the hyperphosphorylation of p100/p120 seen in response to the phosphatase inhibitors calyculin A and okadaic acid (chapter 3, fig 3.8) would be associated with a decrease in permeability. However, these pharmacological agents are unsuitable for analysis of physiological responses as they also caused a disruption of the cytoskeleton (revealed by phalloidin labelling; data not shown), probably as a consequence of increased phosphorylation of many cellular proteins.

I found no difference in p100/p120 behaviour following dephosphorylation. These proteins did not dissociate from E-cadherin, even after prolonged stimulation of PKC. There is no visible disruption of the adherens junctions in response to PDB, as seen by both immunofluorescence, and [35S]methionine labelling of p100/p120 associated proteins. Pulse-chase labelling analysis showed that the stability of
p100/p120 in MDCK I cells is not altered in response to PDB. Thus the effect of p100/p120 dephosphorylation on the function of these proteins is still not clear.
6. PKC and tyrosine phosphorylation

6.1 Introduction

A number of experiments have demonstrated that tyrosine phosphorylation of junctional proteins may be involved in regulating the barrier function. For example, transfection of cells with oncogenic Src or Ras induces tyrosine phosphorylation of components of the adherens junctions, with a concomitant disruption of adhesion (Matsuyoshi et al., 1992; Kinch et al., 1995). Tyrosine phosphatase inhibitors such as vanadate promote tyrosine phosphorylation of both adherens junction and tight junction proteins, and also disrupt the paracellular barrier (Staddon et al., 1995b). PKC can activate signalling pathways that promote tyrosine phosphorylation of certain proteins (see for example Zachary et al., 1993). I investigated the tyrosine phosphorylation of cellular proteins in epithelial cells in response to activation of PKC with PDB, and whether such pathways might be involved in the modulation of intercellular junctions by PKC.

6.2 Phosphorylation of cellular proteins in PDB-treated MDCK I cells

MDCK I cell were treated with 200 nM PDB for 0, 5 or 15 minutes, lysed into sample buffer, and the protein separated by SDS-PAGE. Proteins were transferred to nitrocellulose, and probed with antibody recognising phosphotyrosine residues (fig. 6.1). A number of different cellular proteins show an increase in phosphotyrosine, but the most prominent is a protein with a molecular weight of approximately 170 kD.

Phosphorylation of proteins on tyrosine residues in response to PDB was also analysed in Caco-2, LLC-PK₁ and MDBK epithelial cells. Epithelial cells were treated with PDB for 30 minutes, and proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine antibodies (fig. 6.2). In each of these cells, a protein with an apparent molecular weight of 170kD became
Fig. 6.1 Tyrosine phosphorylation of proteins in MDCK I cells in response to PDB. MDCK I cells were treated with 200 nM PDB for 0, 5 or 15 minutes, then lysed into Laemmli sample buffer, and the protein separated by SDS-PAGE. Following transfer to nitrocellulose, phosphotyrosine-containing proteins were detected by immunoblotting. The mobility of the molecular weight standards are indicated. A number of proteins become phosphorylated on tyrosine in response to PDB, the most prominent being a protein with an apparent molecular weight of 170 kD.
Fig. 6.2 Tyrosine phosphorylation in response to PDB in a variety of epithelial cell lines
Caco-2, MDBK or LLC-PK₁ epithelial cells were grown on polycarbonate filters, treated with 200 nM PDB or DMSO (1:1000) as a control for 30 minutes. Cells were lysed in Laemmli sample buffer, and the proteins separated by SDS-PAGE and transferred to nitrocellulose. Phosphotyrosine was detected by immunoblotting. In each of these cell lines, a protein of about 170 kD become phosphorylated on tyrosine in response to PDB (indicated by arrow). Other protein are also subject to phosphorylation, but only the p170 protein was reproducibly tyrosine phosphorylated in all these cell lines.
phosphorylated on tyrosine in response to PDB.

6.3 p170 solubility

As part of a series of experiments to determine the nature of the p170 protein MDCK I cells treated with increasing concentrations of PDB were lysed into TDS lysis buffer, which contains 1% Triton X100, 0.2% SDS and 0.5% sodium deoxycholate. These relatively harsh detergent conditions serve to solubilise most cellular proteins. The lysates were rotated for 20 minutes, then centrifuged at 14,000 x g. Both supernatant (TDS soluble fraction) and the pellet (TDS insoluble fraction) were then lysed in Laemmli sample buffer, and protein separated by SDS-PAGE. Phosphotyrosine was detected by immunoblotting (fig. 6.3). The tyrosine phosphorylated p170 protein is present exclusively in the TDS insoluble fraction. Analysis of the TDS soluble and insoluble fractions demonstrated that a number of other cellular proteins become tyrosine phosphorylated in response to PDB. Some of these proteins were not detected by anti-phosphotyrosine blotting of whole cell lysates; presumably the enrichment of these proteins that occurred during this procedure was necessary to allow detection.

6.4 Tyrosine phosphorylation of proteins in response to PDB is inhibited by herbimycin A.

Pre-treatment of epithelial cells with the tyrosine kinase inhibitor herbimycin A (Uehara et al., 1989; Weinstein et al., 1991) completely blocks the tyrosine phosphorylation of p170 in response to PDB (fig. 6.4). The TDS insoluble fraction of MDCK I cells was analysed, rather than whole cell lysates, as this fraction is enriched in p170, and so improves ease of detection. Again, use of this fraction allowed detection of tyrosine phosphorylation of other cellular protein in response to PDB in addition of p170. In particular, proteins with apparent molecular weights of 70 and 80
Fig. 6.3 Solubility of p170

MDCK I cells were treated with increasing concentrations of PDB, as indicated, and separated into TDS soluble and insoluble fractions, by centrifugation of the TDS cell lysate at 14,000 g. These fractions were lysed into Laemmli sample buffer, and the proteins separated by SDS-PAGE, and then transferred to nitrocellulose for immunoblotting with anti-phosphotyrosine antibodies. The tyrosine phosphorylated p170 protein is only present in the TDS insoluble fraction (indicated by arrow). Tyrosine phosphorylation of other proteins in response to PDB can also be detected in these fractions. The most prominent of these are proteins of approximately 70 kD and 140 kD present in both the TDS soluble and TDS insoluble fraction, indicated by ( ).
Fig. 6.4 Herbimycin A blocks tyrosine phosphorylation in response to PDB

MDCK I cells grown on polycarbonate filters were treated with increasing concentrations of PDB for 15 minutes, lysed into TDS lysis buffer, and the insoluble fraction harvested to increase yield of the p170 protein. A layer of cells that had been pre-treated for 16 hours with 5 μM herbimycin A was treated with 200 nM PDB for 15 minutes, and the TDS insoluble fraction harvested. These fractions were lysed into Laemmli sample buffer, separated by SDS-PAGE and transferred to nitrocellulose. Ponceau S staining demonstrated equal recovery of proteins (data not shown). Phosphotyrosine was detected by immunoblotting. The tyrosine phosphorylation of p170, and proteins with molecular weight of approximately 70 and 80 kD (indicated by * ) in response to PDB was completely inhibited by Herbimycin A.
kD were tyrosine phosphorylated in cells treated with 100 or 200 nM PDB. The tyrosine phosphorylation of these proteins was also inhibited by pre-treatment of cells with herbimycin A.

6.5 Herbimycin A blocks the increase in paracellular permeability in response to PDB.

The physiological effect of blocking PDB-induced tyrosine phosphorylation with herbimycin A was tested. The resistance across monolayers of MDCK I cells grown on polycarbonate filters was assayed. Where cells had been pre-treated with herbimycin A, the resistance drop in response to PDB was almost completely blocked (fig. 6.5 A). Analysis of \[^{14}C\]sucrose flux showed that this too was blocked by the presence of herbimycin A. Thus, the tyrosine phosphorylation of cellular proteins, including p170, in response to activation of PKC correlates with increased paracellular permeability.

6.6 p170 protein co-migrates with ZO-2.

The tight junction protein ZO-2 has a molecular weight of 170 kD. It can become phosphorylated on tyrosine residues in A431 cells treated with EGF or MDCK I cells treated with the tyrosine phosphatase inhibitors phenyl arsine oxide or pervanadate. Thus ZO-2 might be the p170 protein that becomes tyrosine phosphorylated in response to PDB. Stripping and reprobing a phosphotyrosine-probed filter with antibodies raised against ZO-2 (Jesiatis and Goodenough, 1994) showed that the TDS insoluble fraction contains ZO-2, and that migration of ZO-2 exactly coincided with that of p170 (fig. 6.6).
Fig. 6.5 Herbimycin A blocks PDB-induced permeability increase in MDCK I cells. MDCK I cells grown on polycarbonate filters were treated with or without herbimycin A for 16 hours, then stimulated with PDB at 200 nM. Cells treated with DMSO alone were also assayed as a control. Panel A shows resistance decrease in response to PDB (starting resistance was 4000 Ω.cm²). The resistance drop in response to PDB is attenuated significantly (A). Panel B shows the paracellular[^14C]sucrose flux; pre-treatment of cells with herbimycin A completely abolished the increase in sucrose flux in response to PDB.
Fig. 6.6 ZO-2 co-migrates with p170
Panel A shows a phosphotyrosine probe of proteins in the TDS insoluble fraction of MDCK I cells, treated with increasing concentrations of PDB as indicated. Panel B shows the same filter following stripping, and reprobing with ZO-2 antibody. The position of the 200 kD molecular weight marker is indicated; Overlaying the autoradiograph from the ZO-2 reprobe on the phosphotyrosine blot shows that p170 and ZO-2 migrate to exactly the same position on SDS-PAGE.
6.7 Generation of a peptide-directed ZO-2 antibody

The discovery that ZO-2 co-migrates the p170 protein raises the possibility that these two proteins are the same. ZO-2 antibody was in short supply, as there is no commercially available antibody against ZO-2, so antibodies were generated in order to allow further investigation. Using DNA Analysis software (DNAsstar), two internal peptides from the sequence of dog ZO-2 were identified that had a high surface probability, high hydrophilicity, and a high antigenic index. These peptides are also conserved in the human ZO-2 protein (originally identified as human x104), so any antibody raised against these peptides should show cross-species reactivity. These peptides corresponded to residues 889-901 (MEGMDDDPEDRM) and 966-978 (SPEPRAQMRRAAS). Synthetic peptides containing these two sequences were constructed, with an additional C-terminal lysine residue which is not in the protein to allow coupling to the carrier molecule keyhole limpet haemocyanin (KLH). The peptides were coupled to KLH, and then a mixture of both peptides was injected into rabbits. Two peptides were used to increase the chance of raising an antibody. Antibodies were purified by affinity to peptides bound to sepharose columns.

This procedure generated an antibody (designated pepZO-2) which reacted with a single band at 170 kD in MDCK I cells, the expected molecular weight of ZO-2 (fig 6. 7 A). Similarly, a 170 kD protein was recognised by pepZO-2 in ZO-1 immunoprecipitates from TDS lysis buffer (where ZO-2 remains complexed to ZO-1). In the reciprocal experiment, The pepZO-2 antibody was capable of immunoprecipitating ZO-1 and ZO-2 (as recognised by the original ZO-2 antibody raised against a GST-ZO-2 fusion protein (Jesiatis and Goodenough, 1994). Immunoprecipitation carried out on [35S]methionine labelled MDCK I cell showed that the pepZO-2 antibody specifically purified proteins at 170 and 220 kD, corresponding to ZO-2 and ZO-1 respectively. Immunofluorescence studies showed that the pepZO-2 antibody labelled regions of cell-cell contact, in both canine MDCK and human Caco-2 epithelial cells (fig 6.8). Therefore, the pepZO-2 is a specific antibody against the
Fig. 6.7 Characterisation of pepZO-2 antibody

(A) PepZO-2 antibody recognises a single band of 170 kD in MDCK I whole cell lysates. (B) MDCK I cells were lysed into TDS lysis buffer and immunoprecipitations carried out using the pepZO-2 antibody. Proteins were separated by SDS-PAGE, and transferred to nitrocellulose and probed with pepZO-2, ZO-2 antibody (Jesiatis et al.) or ZO-1 as indicated. The pepZO-2 antibody was capable of immunoprecipitating ZO-2 and ZO-1, which remains complexed with ZO-2 under TDS lysis conditions. Also, ZO-1 immunoprecipitations purified a protein which was recognised by the pepZO-2 antibody. (C) Immunoprecipitations from [35S]methionine labelled MDCK I cell lysates were carried out using the pepZO-2 antibody. A major band at 170 KD was detected, with a 220 kD protein also purified (the expected molecular weight of ZO-1). Immunoprecipitations using non-specific rabbit IgG did not immunoprecipitate these proteins.
Fig. 6.8 Immunoflorescence staining with pepZO-2 antibodies
Immunoflorescence staining shows that pepZO-2 recognises a proteins localised at cell-cell contacts. (A) MDCK I cells, (B) Caco-2 cells.
ZO-2 protein, which recognises both canine and human forms of the proteins, and can be used in western blotting, immunoprecipitation and immunofluorescence.

6.8 The p170 protein that becomes tyrosine phosphorylated in response to PDB is not ZO-2.

MDCK I cells were treated with PDB or DMSO for 30 minutes, and lysed into TDS lysis buffer. The insoluble fraction was obtained by centrifugation, then solubilised in SDS lysis buffer, which contained 1% SDS. The sample was heated at 100°C for three minutes, and passed 10 times through a 23 gauge needle to ensure efficient solubilisation of proteins. The samples was then diluted 1:4 in Triton dilution buffer, and immunoprecipitation with anti-phosphotyrosine antibodies carried out. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against either phosphotyrosine, or ZO-2. This procedure allowed immunoprecipitation of the p170 protein, which co-migrates with ZO-2 (as shown by parallel probing of the SDS-solubilised lysate). However, ZO-2 antibodies did not recognise p170 (fig. 6.9). Since neither of the peptides antigens used to raise the ZO-2 antibody contain a tyrosine residue, this lack of reactivity could not be explained by a disruption of the antibody recognition site by phosphorylation. It was not possible to perform the reciprocal experiment; purifying the TDS insoluble pool of ZO-2 by immunoprecipitation, and probing for phosphotyrosine in a western blot, since the pepZO-2 antibodies were unable to immunoprecipitate ZO-2 under these conditions.

The procedure of solubilising the TDS insoluble proteins in SDS lysis buffer, and then immunoprecipitating phosphotyrosine-containing proteins again showed that a number of proteins become tyrosine phosphorylated in response to activation of PKC in MDCK I cells. More tyrosine phosphorylated proteins were detected than by blotting TDS insoluble fractions alone (compare fig. 6.9 with 6.4); the immunoprecipitation of phosphotyrosine-containing proteins allowed a further enrichment, and this presumably
Fig. 6.9 Immunoprecipitation of tyrosine phosphorylated proteins from the TDS insoluble fraction of MDCK I cells

MDCK I cells were treated with (+) or without (-) 200 nM PDB for 30 minutes, lysed into TDS lysis buffer, and the insoluble fraction purified by centrifugation at 14,000 g. The pellet was resuspended in SDS lysis buffer, then diluted with triton dilution buffer to allow immunoprecipitation to be carried out. (A) A number of tyrosine phosphorylated proteins can be purified by immunoprecipitation under these conditions, including the p170 protein. A reprobe of this blot with ZO-2 antibody showed that this p170 protein is not recognised by ZO-2 antibody. (B) Phosphotyrosine and ZO-2 probe of proteins from the TDS pellet resuspended in SDS/triton dilution buffer. This demonstrated that ZO-2 was present in this fraction, and co-migrated with the tyrosine phosphorylated “p170” protein. It also serves as control to show that the lack of ZO-2 signal in the phosphotyrosine immunoprecipitation shown in panel A was not simply due to a problem in immunoblotting.
increased detection of proteins during immunoblotting. Determining the identity of these proteins will require further investigation.

6.9 Herbimycin A has no effect on the PDB-induced p100/p120 dephosphorylation

The ability of herbimycin A to block the PDB-induced opening of intercellular junctions suggests that the pathway by which PKC activation causes opening of junctions involved a tyrosine kinase (or kinases). I investigated whether the p100/p120 dephosphorylation of p100/p120 was inhibited by herbimycin A. Pre-treatment of MDCK I cells for 16 hours with herbimycin A was followed by addition of DMSO or PDB for 30 minutes. p100 and p120 mobility was analysed by immunoblotting. Herbimycin A did not inhibit the p100/p120 mobility shift (fig. 6.10). Therefore, PKC does not induce p100/p120 dephosphorylation via the herbimycin-inhibitable tyrosine kinase pathway. Also, these results confirm that p100/p120 dephosphorylation alone is not sufficient to cause opening of intercellular junctions, since PKC-dependent p100/p120 dephosphorylation occurs in the presence of herbimycin A, which completely blocks the PKC-dependent increase in paracellular permeability.

6.10 Discussion

The data presented in this chapter provide interesting preliminary evidence that a tyrosine kinase or kinases are involved in the pathway by which PKC opens tight junctions. A number of cellular proteins become phosphorylated in response to PKC. In particular, a 170 kD protein becomes dephosphorylated in response to PKC in a number of different epithelial cell lines. Blocking the tyrosine phosphorylation of cellular proteins, including p170, using the tyrosine kinase inhibitor herbimycin A abolishes the PDB-induced opening of tight junctions, as assayed by paracellular [¹⁴C]sucrose flux. The electrical resistance drop in response to PDB is also attenuated. It is likely that the small resistance drop that still occurs across MDCK I cells in
Fig. 6.10 Herbimycin A does not block p100/p120 dephosphorylation in response to PDB
MDCK I cells were pre-treated for 16 hours with Herbimycin A, or vehicle DMSO as a control, then treated with 200 nM PDB or DMSO (1:1000) for 30 minutes, and the p100/p120 mobility analysed by immunoblotting. Herbimycin A did not inhibit p100/p120 dephosphorylation in response to PDB.
response to PDB in the presence of herbimycin A is due to non-junctional effects, such as activation of ion channels.

The identity of these tyrosine phosphorylated proteins remains to be determined. The most reproducible effect was seen on the p170 protein, which proved not to be the tight junction protein ZO-2. p170 is insoluble even in TDS lysis buffer, suggesting it may be linked to the cytoskeleton. The tyrosine phosphorylation of the other cellular proteins was less reproducible. Some were only seen when cells were fractionated, allowing enrichment of these proteins. Tyrosine phosphorylation of an approximately 70 kD protein was seen in the TDS insoluble fraction (fig. 6.3, 6.4), and this is likely to be paxillin, which becomes tyrosine phosphorylated in response to PKC (Zachary et al., 1993; see also fig. 3.5). Tyrosine phosphorylation of other proteins was also seen in the TDS insoluble fraction, and a number of additional proteins were detected when anti-phosphotyrosine immunoprecipitations were performed on this fraction.

The p100/p120 dephosphorylation in response to PDB is not inhibited by herbimycin A. These data are consistent with a number of different scenarios by which PKC could promote opening of junctions (fig. 6.11). First, p100/p120 dephosphorylation and tyrosine phosphorylation of p170 and other cellular proteins may occur via two distinct pathways in response to PDB. The tyrosine phosphorylation pathway appears to play a key role in opening of intercellular junctions, since inhibition of tyrosine phosphorylation prevents this response. p100/p120 dephosphorylation is clearly not sufficient in itself to cause disruption of junctions, since dephosphorylation of these proteins occurs in the presence of herbimycin A, where opening of intracellular junctions does not occur. Therefore it is possible that p100/p120 dephosphorylation is not involved in disruption of intercellular junctions (fig. 6.11 A). Alternatively, p100/p120 may be necessary to allow disruption of junctions - acting in parallel with the tyrosine phosphorylation pathway (fig. 6.11 B). A further possibility is that p100/p120 dephosphorylation and tyrosine phosphorylation of cellular proteins in response to PDB could occur on the same pathway, with p100/p120 dephosphorylation acting upstream of the tyrosine
Fig. 6.11 Three schematic representations showing how p100/p120 dephosphorylation and tyrosine phosphorylation of cellular proteins may be involved in the regulation of paracellular permeability by PKC

(A) Herbimycin A inhibits tyrosine phosphorylation of p170 and other proteins, and this prevents opening of junctions. p100/p120 dephosphorylation may have no role in disruption of intercellular junctions. (B) Both tyrosine phosphorylation and p100/p120 dephosphorylation act in parallel to induce opening of intercellular junctions. Blocking either pathway would prevent PKC-induced junctional disruption. (C) p100/p120 dephosphorylation occurs upstream of the herbimycin A-sensitive kinase(s) in the disruption of intercellular junctions. Any of these schemes would explain the experimental observations presented in this chapter.
phosphorylation (fig. 6.11 C). At present, it is impossible to distinguish between these three possibilities, since reagents that can specifically block p100/p120 dephosphorylation are lacking.
7. General Discussion

Intercellular junctions are dynamically regulated in multicellular organisms, opening and closing in response to various physiological and pathological stimuli. A number of signalling pathways have been shown to modulate the integrity of these junctions, but the molecular mechanisms responsible for these events are, at present, unknown. One regulator of junctions is protein kinase C, and the discovery that the adherens junctions protein p100 and p120 as targets for this kinase identifies a possible mechanism by which PKC may promote opening of intercellular junctions.

Analysis of $[^{32}P]$phosphate-labelled E-cadherin, β-catenin α-catenin and p100/p120 showed that only the phosphorylation state of p100 and p120 was altered in response to activation of PKC by addition of the phorbol ester PDB. In MDCK I cells, these proteins lost approximately 40% of their phosphate due to dephosphorylation of serine and threonine residues. This caused a dramatic alteration of the mobility of these proteins in SDS-PAGE. The lack of any apparent effect on the other components of the adherens junctions does not prove that there is no change in the phosphorylation of these proteins; small changes such as the dephosphorylation of one or two residues might not be detected, since, with the exception of α-catenin, these proteins are highly phosphorylated, even in resting cells.

The effect of PDB on p100/p120 phosphorylation was shown to be due to activation of PKC, and not some other cellular effect of the phorbol ester. This was demonstrated by the fact that PKC inhibitors bisindolylmaleimide I and Ro 31-8425 could inhibit the PDB-induced dephosphorylation. Also, the p100/p120 mobility shift could be produced by DiC₄, a diacylglycerol analogue, which also activates PKC. 4α-PDB, an analogue of PDB which is unable to activate PKC, did not promote p100/p120 dephosphorylation.

Dephosphorylation of p100 and p120 is rapid, occurring within 5 minutes of addition of PDB. It occurs at a similar time to the disruption of tight junctions in
MDCK I cells (as assayed by drop in resistance across a monolayer). The
dephosphorylation can be reversed rapidly by the inhibition of PKC with
bisindolylmaleimide I, and this reversal correlates with a re-establishment of
paracellular resistance. This reversibility argues against the possibility that the
p100/p120 mobility shift and loss of phosphate is due to proteolysis.

Since PKC is a protein kinase, it must cause p100/p120 dephosphorylation
indirectly, by a pathway that involves at least two steps. The simplest mechanism
would be for PKC to phosphorylate and inhibit a p100/p120 kinase, or for PKC to
directly phosphorylate and activate a p100/p120 phosphatase. A number of kinase
inhibitors induced an increase in electrophoretic mobility of p100/p120, consistent with
dephosphorylation. In contrast, inhibition of cellular serine/threonine phosphatases
caus ed a dramatic decrease in the electrophoretic mobility of these proteins, which is
consistent with increased phosphorylation. The phosphatase inhibitors okadaic acid,
calyculin A and cantharadin, which inhibit protein phosphatases 1 and 2A were able to
promote this hyperphosphorylation, whereas cypermethrin, which inhibits protein
phosphatase 2B, did not. However, although p100 and p120 are substrates for these
enzymes, it is not possible at present to conclude that protein phosphatase 1 and 2A are
responsible for the p100/p120 dephosphorylation in response to PKC. It is clear that in
resting cells the p100/p120 phosphorylation state is regulated by the opposing actions
of a serine/threonine kinase (or kinases) and phosphatase(s) (see fig 3.10 for scheme).

A distinctive selection of kinase inhibitors were capable of promoting a
p100/p120 mobility shift similar to that seen in response to PDB. Staurosporine,
KT5926, Gô 6976 and ML-7 all induced a mobility shift, whereas KN-62, H-7, Gô
7874, bisindolylmaleimide and Ro 31-8425 did not. There may be one p100/p120
kinase, which is sensitive to staurosporine, KT5926, Gô 6976 and ML-7. Alternatively,
there could be more than one p100/p120 kinase, which are sensitive to
selected inhibitors. For example, one kinase could be inhibited by ML-7 and KT5926,
whereas another might be sensitive only to staurosporine and Gô 6976. If there were
more than one kinase, inhibition of either of the p100/p120 kinases would be have to
be sufficient to alter the balance between kinases and phosphatases, and so cause p100/p120 dephosphorylation.

The pathways by which PKC might regulate this serine/threonine phosphorylation cycle were investigated. Phosphopeptide maps of p100 from control and PDB-treated MDCK I cells were compared, in an attempt to identify the serine/threonine residues in p100 that were dephosphorylated in response to PKC. The rationale for this approach was that it might identify a particular protein kinase recognition motif, and thus give a clue as to the identity of the p100/p120 kinase. However, the phosphopeptide maps produced were extremely complicated, with the generation of a large number of phosphopeptides. Some differences in phosphopeptide mobility were seen, but it was not possible to identify where in the protein these might be. A decrease in intensity of the $^{32}$P-signal from many of the phosphopeptides generated from p100 was observed, which suggests that perhaps a large number of residues become dephosphorylated in response to PKC activation. During the immunoprecipitation of [$^{32}$P]phosphate-labelled p100/p120, some protein proteolysis occurred, generating 70 kD protein. These fragments were subject to dephosphorylation in response to PDB, and were recognised by the pp120 antibody, which was raised against an epitope close to the C-terminal region of p100/p120. Therefore at least some of the serine/threonine residues that become dephosphorylated in response to PDB are located in the C-terminal two-thirds of p100/p120.

A second approach tested the possibility that known signalling pathways were responsible for p100/p120 dephosphorylation in response to PKC activation. An attractive candidate for the p100/p120 kinase was GSK-3β. This enzyme had been shown to phosphorylate β-catenin, which shares some sequence homology with p100/p120, since both are members of the Armadillo family of proteins. In addition, this kinase can be phosphorylated and inhibited by PKC, at least in vitro. However, GSK-3β does not appear to be the kinase involved in the phosphorylation of p100/p120. Analysis of GSK-3β purified from PDB-treated cells showed that, at least in MDCK I cells, GSK-3β did not appear to be inhibited by activation of PKC.
Recombinant GSK-3β was incapable of phosphorylating p100 and p120 in vitro, even though this enzyme could phosphorylate β-catenin under identical conditions. Treatment of MDCK I cell with kinase inhibitors had shown that KT5926, Gö 6976 and staurosporine cause a p100/p120 mobility shift, but bisindolylmaleimide I and Ro 31-8425 did not. These data implied that the p100/p120 kinase (or kinases) should be inhibited by KT5926, Gö 6976 and/or staurosporine, but not by bisindolylmaleimide I and Ro 31-8425. Analysis of the effect of these kinase inhibitors on the ability of recombinant GSK-3β to phosphorylate a peptide substrate showed that although staurosporine and Gö 6976 did inhibit GSK-3β, KT5926 did not. The PKC inhibitors bisindolylmaleimide I and Ro 31-8425, which have no effect on p100/p120 mobility in cells, were potent inhibitors of GSK-3β. These data argue against the likelihood that GSK-3β is the p100/p120 kinase. Recently, a particular GSK-3β consensus motif towards the N-terminus of β-catenin was shown to be necessary its degradation via the ubiquitin pathway (Aberle et al., 1997). This motif is similar to one found in the N-terminus of IκB, which is also necessary for the ubiquitin-mediated degradation of this protein. p120 does not have this particular GSK-3β consensus motif, which may explain why p100/p120 do not appear to be substrates for GSK-3β.

The MAP kinase pathway can be activated by PKC, and analysis of the tyrosine phosphorylation state of Erk-1 and Erk-2 showed that this pathway was activated in MDCK I cells in response to PDB. However, this pathway could be down-regulated by the MAP kinase kinase inhibitor PD 98059, and yet this inhibitor had no effect on the induction of p100/p120 dephosphorylation in response to PDB. Also, activation of the MAP kinase pathway could be induced by EGF, but EGF did not promote p100/p120 dephosphorylation. Thus PKC does not appear to exert its affects on p100/p120 via the MAP kinase pathway. However, a small activation of Erk-1 and Erk-2 still occurred in response to PDB, even in the presence of PD 98059, so the possibility that this pathway is involved in p100/p120 dephosphorylation in response to PKC cannot be entirely ruled out.
Disruption of E-cadherin-mediated homotypic interaction at the adherens junction by chelation of extracellular Ca\(^{2+}\) did not lead to p100/p120 dephosphorylation. Neither did it inhibit the PDB-induced dephosphorylation. Thus the pathway by which PKC promotes p100/p120 dephosphorylation remains intact, even where the adherens junctions and tight junctions have been disrupted.

Despite the fact that dephosphorylation of p100/p120 was enough to dramatically alter the mobility of these protein during SDS PAGE, there was no corresponding alteration in the behaviour of these proteins in the cell. There was no change in the interaction of these proteins with the adherens junction complex; \([35S]\)-methionine labelling of p100/p120 associated proteins revealed no change in these associations in response to PKC activation, and western blotting confirmed that E-cadherin and \(\beta\)-catenin remain associated with dephosphorylated p100/p120. However, there may be other proteins associated with p100/p120 in cells, which do not remain associated during immunoprecipitation of p100/p120, even under mild detergent conditions. Any alteration in association with these proteins following p100/p120 dephosphorylation would not have been detected in these experiments. There was also no alteration in the stability of p100 and p120 in MDCK I cells following PDB-stimulated dephosphorylation, as shown by pulse-chase labelling.

The absence of any obvious change in p100/p120 behaviour makes predictions about the role of this pathway in cells difficult. Since PKC promotes opening of intercellular junctions, and the p100/p120 associated with the adherens junctions complex becomes dephosphorylated in response to PKC activation, it is possible that p100/p120 dephosphorylation is involved in this pathway. In MDCK I cells, this dephosphorylation occurs rapidly, and indeed precedes effects on paracellular permeability. Rephosphorylation of p100/p120 is followed by a re-establishment of paracellular resistance. The p100/p120 mobility shift occurred in response to activation of PKC in all epithelial cell lines tested, and so may be a common feature of epithelial cells. In LLC-PK\(_1\), Caco-2 and MDCK II cells, this mobility shift is accompanied by an increase in paracellular \([14C]\)sucrose flux, indicating a disruption of tight junctions.
The observation that PKC activation promotes dephosphorylation in endothelial cells and fibroblasts as well as epithelia is interesting. These cells also have cadherin-mediated intercellular adhesion, and cadherin is associated with p120 isoforms in these cells (Takeichi, 1992; Reynolds et al., 1996). Therefore it is possible that dephosphorylation of p100/p120 could be part of the pathway by which junctions are disrupted in many different cell types. p100/p120 dephosphorylation is also observed in human umbilical vein endothelial cells in response to histamine and thrombin (Jim Staddon, personal communication). Histamine and thrombin are inflammatory mediators, and promotes disruption of intercellular junctions. Thus p100/p120 dephosphorylation is involved in a number of signalling pathways which impinge on junctions.

Although a number of signalling pathways can cause disruption of junctions, or promote assembly of junctions, the molecular mechanisms by which this regulation is achieved are at present unknown. Only in the last few years has evidence been presented showing that junctional proteins are targets for signalling pathways. Cadherins, β-catenin and p120 are all substrates for tyrosine kinases, and their phosphorylation on tyrosine correlates with a disruption of intercellular adhesion. Similarly, tyrosine phosphorylation of ZO-1 and ZO-1 correlates with disruption of junctions. However, tyrosine phosphorylation of these proteins has generally been achieved by rather extreme measures; transfection of cells with oncogenic tyrosine kinases (Matsuyoshi et al., 1991), EGF stimulation of A431 cells (which overexpress EGF receptor (Van Itallie et al., 1995)), or use of tyrosine phosphatase inhibitors (Staddon et al., 1995). Only p120 has been shown to be tyrosine phosphorylated in response to more physiological activators, such as PDGF, EGF or CSF-1 (Downing and Reynolds, 1991), but it is not clear that these pathways affect intercellular junctions. β-catenin is a substrate for GSK-3β, a serine/threonine kinase, but this pathway is involved in the Wnt signalling pathway, and appears to be independent of the function of β-catenin at the adherens junction (Orsulic and Piefer, 1996b). Recently, the tight junction component occludin has been shown to become more
phosphorylated as MDCK I cells are induced develop junctions by the addition of Ca\(^{2+}\), and this correlates with recruitment of occludin in to tight junctions (Sakakibara et al., 1997). Yet there are very few examples of intercellular junctions as targets for endogenous pathways which cause disruption of junctions. In this context, the discovery that p100 and p120 are targets for PKC, an intracellular messenger that has a clear role in disruption of junctions is particularly interesting. However, at present, the data linking PKC-induced p100/p120 dephosphorylation to disruption of intercellular adhesion is still only correlative.

If the phosphorylation state of p100/p120 alone is a key regulator of permeability it would be expected that selective manipulation of phosphorylation should give predictable changes in permeability. The hyperphosphorylation of p100/p120 seen in response to the phosphatase inhibitors such as calyculin A and okadaic acid (fig. 3.8) might be expected to block the permeability increase in response to PKC. However, these pharmacological agents are unsuitable for analysis of physiological responses as they also caused a disruption of the cytoskeleton, probably as a consequence of increased phosphorylation of many cellular proteins. Where p100/p120 dephosphorylation was induced by the kinase inhibitors Go 6976 and KT5926, this was not associated with increased paracellular sucrose flux (fig. 5.5). One interpretation is that p100/p120 dephosphorylation is involved in permeability regulation but additional effects of KT5926 and Go 6976 on other kinases prevent the physiological response. Another is that p100/p120 dephosphorylation alone is not sufficient to trigger a permeability increase. It is likely that PKC activation acts to increase tight junction permeability by concerted regulation of a number of events, perhaps including p100/p120 dephosphorylation.

Other potential roles for p100/p120 in signalling exist. There is a significant pool of p100/p120 that does not appear to interact with cadherins which also undergoes dephosphorylation in response to PDB stimulation. This p100/p120 pool may have some cellular function completely separate from any role in cell adhesion. PKC family members have many diverse roles in the cell: control of growth and proliferation,
control of gene expression, regulation of cell morphology and cell-matrix adhesion are just a few examples (Kikkawa and Nishizuka, 1986; Nishizuka, 1986). p100/p120 dephosphorylation may be involved in any of these processes. Another member of the Armadillo family, β-catenin, has been shown to have roles in cell signalling pathways independent from its classical role in cell adhesion. β-catenin participates in the Wnt signalling pathway and interacts with the adenomatous polyposis coli (APC) gene product (Rubinfeld et al., 1993; Su et al., 1993), as well as the transcription factors LEF-1 (Behrens et al., 1996) and XTcf-3 (Molenaar et al., 1996). It is possible that p100 and p120 may also prove to have multiple functions. Overexpression of p120 in NIH 3T3 fibroblasts caused cells to elongate, and extend dendritic processes (Reynolds et al., 1996), which suggests a possible function for p120 in regulation of the cytoskeleton. However, overexpression of p120 in epithelial cells had little effect on morphology, and the physiological relevance of the effect on morphology in fibroblasts is unclear. Determination of the biological significance of p100/p120 serine/threonine dephosphorylation will require further investigation. Future experiments should focus on selective regulation of p100/p120 phosphorylation to clarify these issues. Potential areas of research include expression and purification of p120 protein to use as a substrate for in vitro kinase assays, which may allow purification of the p100/p120 kinase.

The data on tyrosine phosphorylation in response to PDB are rather preliminary, but provide an interesting avenue for further investigation. Tyrosine phosphorylation of junctional proteins had been implicated in the disruption of intercellular adhesion. However, as mentioned above, this had only been done under non-physiological conditions; transfection of cell with oncogenic Src or oncogenic Ras, or use of tyrosine phosphatase inhibitors. Activation of PKC with phorbol ester provides a much closer approximation to a physiological event. I found good correlation between tyrosine phosphorylation of cellular proteins and disruption of tight junctions; blocking tyrosine phosphorylation of these protein in response to PDB using herbimycin A completely blocks the disruption of the paracellular barrier. A large number of cellular proteins are
phosphorylated on tyrosine in response to PKC. Analysis of whole cell lysates, TDS insoluble fractions, and phosphotyrosine immunoprecipitations revealed different patterns of tyrosine phosphorylation in response to PKC. This is likely to be due to specific enrichment of different proteins under different conditions, which then allowed detection of these tyrosine phosphorylated proteins in immunoblotting. If proteins are not suitably enriched, then the sensitivity of phosphotyrosine immunoblotting appears to be insufficient to allow detection.

The most reproducibly phosphorylated protein was the p170 protein. Tyrosine phosphorylation of a p170 protein was seen in a number of different epithelial cell lines. The TDS insoluble nature of p170 suggests it may be associated with the cytoskeleton. This protein was found not to be ZO-2 by western blotting of phosphotyrosine immunoprecipitated proteins, and the identity of p170 remains at present unknown.

Any one of the proteins which become tyrosine phosphorylated in response to PDB could involved in increasing permeability. It should also be noted that the RC20H anti-phosphotyrosine antibody which was used in the experiments was not particularly sensitive, and enrichment of fractions either by harvesting the TDS insoluble fraction from a large number of cells, or immunoprecipitating phosphotyrosine-containing proteins from this TDS-insoluble fraction was necessary to allow detection of some tyrosine phosphorylated proteins. It is therefore possible that the effect of herbimycin A on permeability could be due to inhibition of tyrosine phosphorylation of a protein that was not detected in the assays described here. The next step will be to identify the proteins which become tyrosine phosphorylated in response to PKC in epithelial cells.

In conclusion, I have identified two PKC-activated signalling pathways that may be involved in regulation of paracellular permeability. First, induction of tyrosine phosphorylation of cellular proteins in response to PKC plays a key role in the opening of cellular tight junctions. Second, the adherens junction proteins p100 and p120 are capable of cycling between higher and lesser phosphorylated forms, subject to the
actions of serine/threonine phosphatase(s) and a serine/threonine kinase(s), and this
cycle is regulated by PKC. The pathway by which PKC promotes p100/p120
dephosphorylation occurs in epithelia, endothelia and fibroblasts. The serine/threonine
phosphorylation cycle is novel and surprising, given the original identification of p120
as a tyrosine kinase substrate. Further investigation of the players involved in
regulation of this cycle will be needed before the physiological importance of
p100/p120 dephosphorylation can be determined. However, these observations open
up new lines of inquiry in the field of cadherin-mediated adhesion.
8. References


Guyton, A. C., and J. E. Hall. 1996. *Text book of Medical Physiology, W.B. Saunders company*


Peifer, M., P. D. McCrea, K. J. Green, E. Wieschaus, and B. M. Gumbiner. 1992. The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the


187


Stanley, J. R., L. Koulu, V. Klaus-Kovtun, and M. S. Steinberg. 1986. A monoclonal antibody to the desmosomal glycoprotein desmoglein I binds the same polypeptide as human autoantibodies in pemphigus foliaceus. *J. Immunology* **136**: 1227-1230.


