A STUDY OF KERATINOCYTE DIFFERENTIATION AND ADHESION *IN VITRO*

by

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ABSTRACT

One of the most widely used techniques to control keratinocyte differentiation *in vitro* is by varying the extracellular calcium concentration - low extracellular calcium concentrations suppress differentiation whereas high concentrations induce differentiation. In this study, I used the serum-free MCDB 153 culture system to investigate calcium-induced keratinocyte differentiation.

Treating normal human keratinocytes with high extracellular calcium concentrations (1mM) increased the proportion of cells expressing differentiation-specific proteins. I showed that this was not caused by calcium-induced cell-cycle arrest, nor was it a consequence of stratification. However, the expression of differentiation-specific proteins was preceded by the formation of cadherin-mediated cell-cell adhesions.

The likely importance of the cadherin-mediated adhesions in initiating the differentiation program was confirmed in two ways. Firstly, clustering cell-surface E-cadherin in low extracellular calcium using monoclonal antibodies increased the proportion of keratinocytes expressing differentiation-specific proteins. Secondly, suppressing the formation of cadherin-mediated cell-cell adhesions using synthetic peptides analogous to the cadherin recognition domain attenuated the calcium-induced expression of differentiation-specific proteins. These data are consistent with a role for the cadherin-mediated cell-cell adhesions in initiating the keratinocyte differentiation program in response to calcium *in vitro*.

A second aspect of this project involved an investigation of the role played by the Src-family of protein tyrosine kinases at calcium-induced cadherin-mediated adherens junctions. The ubiquitously expressed members of this family, c-Src, Fyn and c-Yes were localised to the cadherin-mediated adhesions formed in response to high extracellular calcium. Treating adherent keratinocytes maintained in low extracellular calcium with a specific Src kinase inhibitor, PD162531, induced the assembly of cadherin-mediated cell-cell adhesions leading to the formation of contiguous groups of cells similar to those seen in response to high extracellular calcium. The inhibitor did not affect the formation of cadherin-mediated adhesions in response to high extracellular calcium, nor did it affect the induction of differentiation-specific proteins under these conditions.

From this study, I conclude that the cadherin-mediated cell-cell adhesions are the likely origin of the signal which initiates keratinocyte differentiation in response to high extracellular calcium *in vitro*. The data presented are consistent with a role for the Src kinases in regulating adherens junction turnover but do not exclude a role also in modulating aspects of differentiation.

To Mum and Dad

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Declaration.

I declare that all the work in this thesis was performed personally unless otherwise acknowledged

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ABBREVIATIONS

Standard abbreviations for SI units and chemical names are not listed

APC ....adenomatous polyposis coli
APS ....ammonium persulphate
ATP ....adenosine 5′-triphosphate
BCA ....bicinchoninic acid
BPE ....bovine pituitary extract
BSA ....bovine serum albumin
c- ....cellular
cpm ....counts per minute
Da ....daltons
DAB ....3,3′-diaminobenzidine tetrahydrochloride
DAPI ....4′,6-diamidino-2-phenylindole
DMSO ....dimethylsulphoxide
DNA ....deoxyribonucleic acid
ECL ....enhanced chemiluminescence
ECM ....extracellular matrix
EDTA ....ethylenediamine tetraacetic acid, disodium salt
EGF ....epidermal growth factor
EGTA ....ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid
ELISA ....enzyme-linked immunosorbent assay
FCS ....foetal calf serum
FITC ....fluorescein isothiocyanate
GSK3β ....glycogen synthase kinase 3β
GTP ....guanosine 5′-triphosphate
HEPES ....N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]
HKGM ....high calcium keratinocyte growth medium
HPV ....human papilloma virus
Ig ....immunoglobulin
KBM ....keratinocyte basal medium
KGM ....keratinocyte growth medium
LKGM ....low calcium keratinocyte growth medium
mAb ....monoclonal antibody
MDCK ....madin-darby canine kidney
MEM ....modified eagle medium
OD at x ....optical density, where x is the wavelength
PAGE ....polyacrylamide gel electrophoresis
PBS ....phosphate buffered saline
PIPES ....piperazine-N,N′-bis[2-ethanesulphonic acid]
PKC ....protein kinase C
PMA ....Phorbol 12-myristate 13-acetate
PNA ....peanut agglutinin
PMSF ....phenylmethylsulphonyl fluoride
RNA ....ribonucleic acid
SCC ....squamous cell carcinoma

SDS ....sodium dodecyl sulphate
SEM ....standard error of the mean
TCA ....trichloroacetic acid
TdT ....terminal deoxynucleotidyl transferase
TEMED........N,N,N′,N′-tetramethylethylenediamine
TGk ....keratinocyte transglutaminase
Tris ....tris-[hydroxymethyl]aminomethane
TRITC ....tetramethyl rhodamine isothicyanate
TUNEL ....TdT-mediated dUTP-biotin nick end labelling
TXi ....Triton X100-insoluble
TXs ....Triton X100-soluble
UTP ....uridine 5′-triphosphate
v- ....viral
VCFS ....velo-cardio-facial syndrome
w/v ....weight for volume
w/w ....weight for weight

ABBREVIATIONS FOR AMINO ACIDS:

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	X

Throughout this study, the names of genes are presented in italics (e.g. *fyn*) and the names of proteins are given in normal text beginning with a capital letter (e.g. Fyn).

INTRODUCTION

CHAPTER 1
THE KERATINOCYTE AND THE MAMMALIAN EPIDERMIS

The keratinocyte is the principal cell type of the outer surface of mammalian skin, the epidermis. The skin is an organ of crucial biological importance, providing a physical barrier between the internal *milieu* of the animal and the surrounding environment, impeding water exchange and providing protection against pathogenic organisms and physical insults. These are functions to which the epidermis and the highly specialised keratinocyte are remarkably well-adapted.

1.1 THE STRUCTURE OF THE EPIDERMIS

The epidermis is a stratified structure spanning the distance between the basal lamina at the interface with the dermis, and the outermost surface of the organism. It is subdivided into series of layers or strata which are distinguished histologically and correspond to different stages in the maturation of the keratinocyte, with the most specialised, or differentiated, cells on the outer surface of the epidermis. Proliferation takes place exclusively in the deepest (basal) layer and the cells generated here are subsequently displaced outwards to begin their journey towards the outermost (cornified) layer, a process that takes 10 to 14 days (Epstein and Maibach, 1965) in human skin culminating in the production of tough, enucleate squames or corneocytes with no metabolic activity. These highly specialised structures knit together to give the epidermis a tough impermeable outer surface.

1.1.1 The layers of the epidermis

A. The basal layer

The basal layer in normal adult epidermis (Figure 1 *a*) is restricted to a single layer of columnar cells immediately adjacent to the basal lamina at the dermal-epidermal junction. It is believed that the unipotent stem cells which ultimately generate all the keratinocytes of the epidermis reside within this basal layer (Jones *et al.*, 1995), constantly producing keratinocytes *via* a transit-amplifying stage. The products of this proliferative process eventually become the post-mitotic cells of the basal layer that are

Figure 1

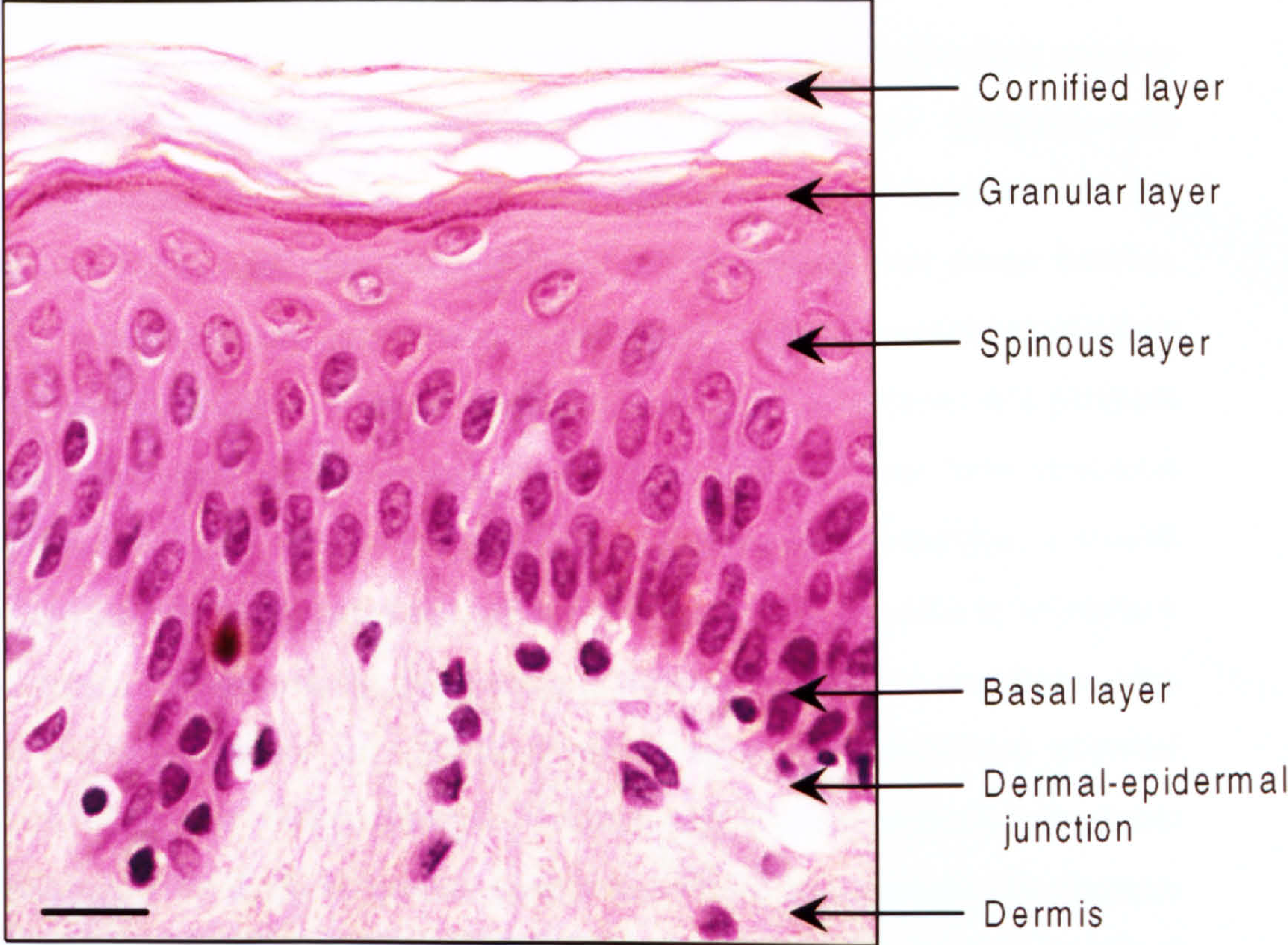
Figure 1
The structure of human epidermis

a. Bright-field photomicrograph showing a transverse section of human skin indicating the principal strata of the epidermis. The single layer of proliferative basal cells is adjacent to the paler-coloured dermis, beneath the spinous layer which consists of multiple layers of cells. The darker granular layer consists of a thin layer of flattened cells and is followed by the thicker cornified layer of enucleate corneocytes. No hair follicles are visible in this preparation. Haematoxylin and eosin stain. Scale bar represents 20μm. This tissue preparation was provided by the pathology department at Ysbyty Gwynedd, Bangor.

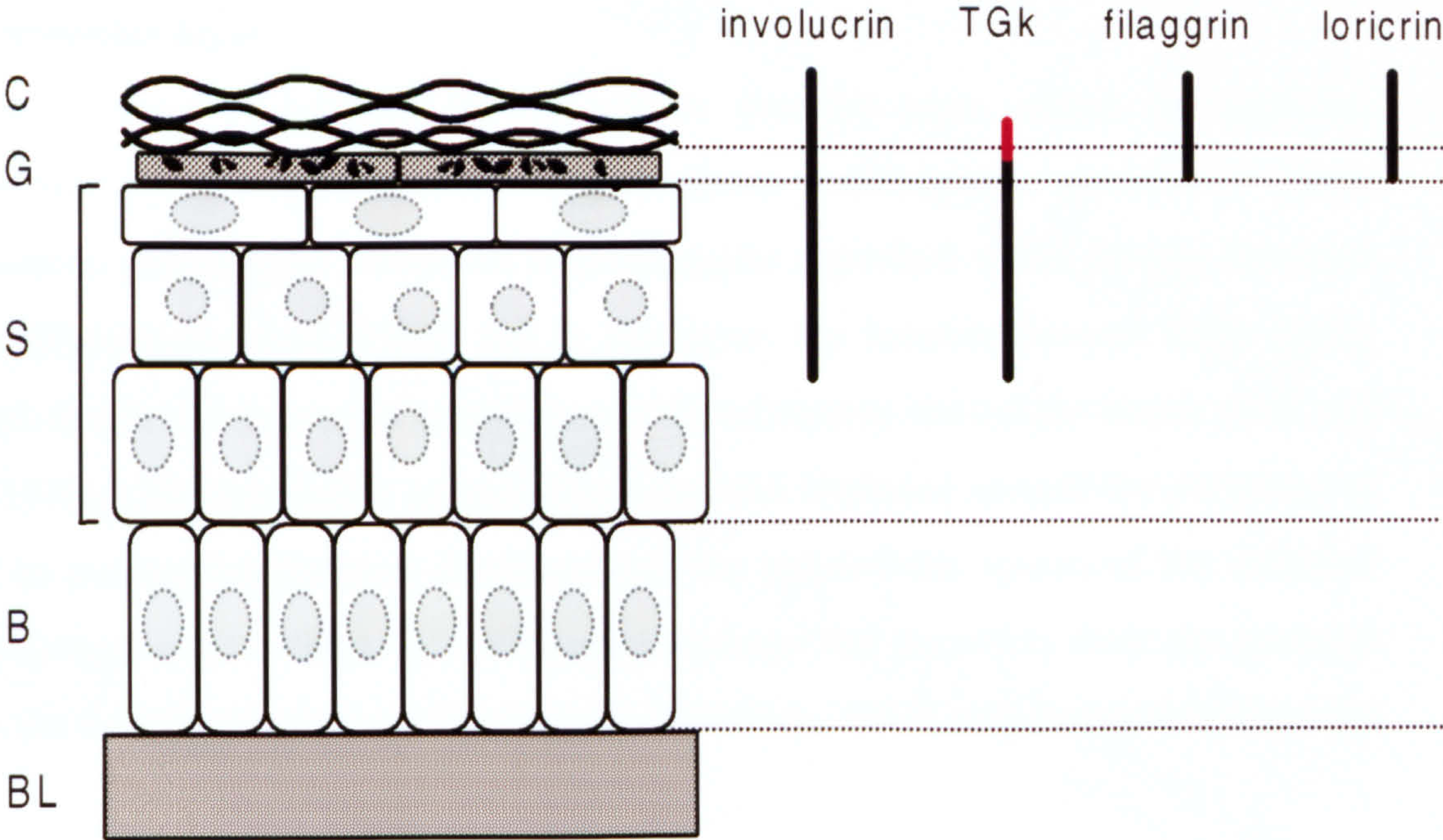
b. Schematic diagram representing the strata of the epidermis spanning the distance between the basal lamina (BL, exaggerated for clarity) and the surface of the skin, showing where some of the envelope proteins expressed by differentiating keratinocytes are detectable (black bars). The red bar indicates where keratinocyte transglutaminase (TGk) is activated during the transition between the granular and cornified layers. B, basal layer; S, spinous layer; G, granular layer and C, cornified layer.

The structure of human epidermis

a. **Human epidermis (transverse section)**



b. **Differentiation-specific protein expression in human epidermis**



committed to the differentiation program. Thus, the epidermis is maintained in a state of dynamic self-renewal, with continuous proliferation in the basal layer replacing the keratinocytes that are shed from the outermost cornified layer.

B. The spinous layer

The next layer, closer to the surface of the epidermis, is the spinous layer which consists of multiple layers of keratinocytes becoming progressively more flattened with increasing distance from the basal layer. In the spinous layer, the synthesis of new keratins (section 1.2.2) begins and these proteins are assembled into dense bundles giving the cells a characteristic appearance when visualised by transmission electron microscopy. In addition to these keratins, keratinocytes begin the synthesis of a plethora of new proteins upon entering the spinous layer. A number of these have structural roles and contribute in some way to the assembly of the cornified envelope, a crucial component of the fully differentiated keratinocyte (section 1.2.1). In addition to changes in protein synthesis, changes in the lipid composition of the plasma membrane also begin to take place (Lampe *et al.,* 1983), contributing to the specialised physical properties of the differentiated keratinocyte. Intracellular membrane-bound structures containing a mixture of lipid, carbohydrate, protein and hydrolytic enzymes also become visible in the upper spinous layer. The contents of these lamellar granules are involved in waterproofing the outer surface of the epidermis when they are discharged into the intercellular spaces late in the differentiation process (Holbrook, 1989).

C. The granular layer

The spinous layer is followed by the narrow granular layer, where the cells are characterised by the presence of electron-dense keratohyalin granules. These proteinaceous granules are composed of profilaggrin deposited where keratin filaments intersect (Fukuyama *et al.,* 1980) and in this layer, the keratins become more highly stabilised due to the increased prevalence of interfilament disulphide bonds (Sun and Green, 1978). The lammellar granules now become clustered around the periphery of the cell in preparation for their discharge into the intercellular spaces of the cornified layer (Madison *et al.,* 1988). Keratinocytes undergo the transition from the granular layer to the cornified layer in about 6 hours (Matoltsy, 1986) and during this time, the

plasma membrane becomes permeable to calcium ions (Ca^{++}), the organelles and nucleus are destroyed and the cornified envelope is assembled (section 1.2.1).

D. The cornified layer

The outermost layer of the epidermis, the cornified layer, consists of multiple layers of interlocking enucleate cornified keratinocytes (corneocytes) and varies considerably in thickness depending, for example, upon its location on the body. The metabolically inactive corneocytes of this layer have fully assembled cornified envelopes and are sealed in the waterproof intercellular lipid matrix discharged by the lamellar granules (Potts and Francour, 1991). As the corneocytes approach the surface of the epidermis, they become less tightly associated with one another and finally, are shed into the environment.

1.1.2 Non-keratinocytes in the epidermis

Although the keratinocyte is the principal cell type in the epidermis, other cell types are also present. These include melanocytes, langerhans cells and merkel cells. Melanocytes produce the photoprotective pigment, melanin, which is contained in membrane-bound melanosomes which are transferred to basal keratinocytes by a variety of mechanisms (Yamamoto and Bhawan, 1994). The langerhans cells are mainly found in the spinous layer and are involved in the uptake, processing and presentation of antigen (Streilein and Bergstresser, 1984) whilst merkel cells are thought to have a variety of different functions including mechanosensation (Iggo, 1985).

1.2 KERATINOCYTE DIFFERENTIATION

1.2.1 The cornified envelope

The cornified envelope is a tough proteinaceous structure which is present immediately below the plasma membrane of the corneocytes and gives these fully differentiated keratinocytes some of their unique properties, being insoluble under all but the most severe conditions (Sun and Green, 1976). The envelope accounts for 10% of the mass of the cornified keratinocyte (Reichert *et al.,* 1993) and its assembly is one of the final

stages of keratinocyte differentiation, taking place at the transition between the granular layer and the cornified layer.

A. The components of the cornified envelope

The cornified envelope is constructed from a variety of proteins which are progressively synthesised during the transit of the keratinocyte from the basal layer to the upper layers of the epidermis (Figure 1 *b*). One of the first envelope components to be identified was involucrin, a 98kDa cytosolic protein that begins to accumulate in the spinous layer of the epidermis, early in the differentiation process (Rice and Green, 1979; Banks-Schlegel and Green, 1981). Another important envelope precursor is filaggrin, a component of the intracellular granules of the granular layer keratinocyte (Lynley and Dale, 1983). Filaggrin is involved in bundling keratin filaments (Dale *et al.,* 1978) and has been identified as an envelope component in both rat and human keratinocytes where it may be involved in linking the keratin intermediate filaments to the envelope (Richards *et al.,* 1988; Steinert and Marekov, 1995). Profilaggrin is synthesised in the granular layer as a phosphorylated 400kDa protein consisting of multiple 45kDa filaggrin repeats (Lonsdale-Eccles *et al.,* 1984) and is then dephosphorylated and cleaved to release filaggrin during differentiation prior to its incorporation into cornified envelopes (Resing *et al.,* 1985).

Other envelope components include loricrin, which accumulates in the granular layer (Mehrel *et al.,* 1990); the pancornulins (Philips *et al.,* 1990); Spr1 (small proline rich protein) (Kartasova and van de Putte, 1988) which accumulates in the spinous and granular layers; keratolinin which first becomes detectable in the spinous layer (Zettergren *et al.,* 1984) and proteins of 195 and 210kDa (Simon and Green, 1984; Ma and Sun, 1986) that were recently cloned and named periplakin and envoplakin respectively (Ruhrberg, 1997 and Ruhrberg *et al.,* 1996).

B. The assembly of the cornified envelope

The various components of the envelope are synthesised progressively as the keratinocyte nears the surface of the skin, but their assembly into the envelope itself is rapid and one of the final stages of the differentiation process. The principal enzyme involved in the assembly of cornified envelopes is the 90kDa membrane-bound keratinocyte transglutaminase (TGk) which catalyses the formation of covalent

isopeptide bonds between glutamine residues and a suitable amine donor such as lysine (Folk and Chung, 1973). These ε-(γ-glutamyl) lysine bonds are formed between the membrane-bound envelope components and the cytosolic envelope components such as involucrin, a preferred substrate for TGk (Simon and Green, 1985; Rorke and Eckert, 1991). TGk is not present in detectable amounts in basal keratinocytes and is first expressed in the suprabasal layers at a similar time to involucrin (Thacher and Rice, 1985) although it remains inactive until later in the differentiation process.

Transglutaminases are enzymes that require the presence of calcium ions (Ca^{++}) for their full activity (Ogawa and Goldsmith, 1976). This is achieved during physiological differentiation by an increase in the permeability of the plasma membrane in the late granular layer allowing the influx of extracellular Ca^{++}, a cation that is at a relatively high concentration in this part of the epidermis (Menon *et al.,* 1985). The process of envelope assembly is believed to begin with the interchain cross-linking of involucrin to itself which then provides a matrix to which other components such as loricrin and the small proline-rich proteins are cross-linked (Steinert and Marekov, 1997). This coordinated assembly process gives an envelope that is rich in involucrin adjacent to the plasma membrane but richer in loricrin and other components on its cytoplasmic face.

1.2.2 The keratins

The keratins are a family of intermediate filament proteins that give the keratinocyte its name and collectively account for up to 30% of the total cellular protein in the basal layer and up to 70% in the cornified layer (Sun and Green, 1978). The keratin intermediate filaments provide keratinocytes with mechanical resilience and allow force to be transmitted through the epidermis *via* the specialised adhesion structures in the plasma membrane with which they interact (Staehelin, 1974). These adhesions, the desmosomes and hemidesmosomes (sections 2.2.1 and 2.1.1) interact with other keratinocytes and with the basement membrane respectively.

A. The structure of the keratins

The keratin family is subdivided into two groups, the type I or acidic keratins and the type II or basic keratins. At least 16 type I and 14 type II keratins, sharing the same basic structure of a central α-helical rod domain and more variable looped head and tail

domains, have been identified (Hanokoglu and Fuchs, 1983). In the extended keratin filaments, one type I and one type II keratin form linear heterodimers (Hatzfeld and Weber, 1990) which then polymerise to form elongated filaments 10nm in diameter. It is thought that phosphorylation of the head region of the keratins may play a role in regulating the formation of these polymers (Eriksson *et al.,* 1992). Different type I and type II keratins can form heterodimers with one another *in vitro* (Hatzfeld and Franke, 1985), but the keratin pairings are normally more ordered. For example, keratin K5 associates with K14 in the basal keratinocytes of normal skin and the differentiation-specific keratins K1 and K10 associate in suprabasal keratinocytes.

B. Changes in keratin expression during keratinocyte differentiation

As alluded to above, the range of keratins expressed by keratinocytes changes during differentiation. Keratins K5 and K14 are specific to the basal layer of adult epidermis and they replace K8 and K18 that are expressed in the skin earlier in development (Byrne *et al.,* 1994) and in simple stratified epithelia (Cooper *et al.,* 1985). In suprabasal keratinocytes, K5 and K14 are no longer expressed and synthesis switches to K1 and K10 (Fuchs and Green, 1980; Moll *et al.,* 1982), although the functional consequences of this are not known. K5 and K14 are stable and remain detectable in the keratinocyte during its transit through the epidermis (Stoler *et al.,* 1988), suggesting that the K1/K10 and K5/K14 pairings may have distinct functions. In the epidermis, some keratin pairs are never normally expressed, for example K6 and K16. However, they are expressed in hyperproliferative psoriatic epidermis and are useful markers of abnormal keratinocyte proliferation and differentiation (Leigh *et al.,* 1995). In addition, the simple epithelial keratins K8 and K18 can be expressed in metastatic squamous cell carcinoma (Markey *et al.,* 1991) indicating that a loss of squamous differentiation has taken place.

1.3 THE AIMS OF THIS STUDY

The original aim of this study was to define the role played by the ubiquitously expressed Src-family kinases c-Src, Fyn and c-Yes in calcium-induced human keratinocyte differentiation *in vitro*. When this study was initiated, two reports describing changes in the activity of c-Src and c-Yes during calcium-induced keratinocyte differentiation had been published (Zhao *et al.*, 1993 and Zhao *et al.*, 1992). These studies analysed a late stage of differentiation, corresponding to the transition between the granular and cornified layers which can only be induced *in vitro* by simultaneous treatment with high extracellular calcium and ionophore (see also section 6.3). This non-physiological manipulation allows the influx of calcium ions, activating the keratinocyte transglutaminase (TGk) thereby leading to the assembly of cornified envelopes by those cells competent to do so. However, high extracellular calcium concentrations in the absence of ionophore are sufficient to induce the earlier stages of differentiation, corresponding to the basal to spinous and spinous to granular layer transitions. The origin of the differentiation-inducing primary signal elicited by treatment with high extracellular calcium alone is unknown. For this study, it was therefore necessary to define the origin of this signal before proceeding with an analysis of the function of the Src-family kinases.

We now believe that it is the formation of cadherin-mediated adherens junctions which is the origin of the differentiation-inducing signal elicited by high extracellular calcium. The remaining two chapters of this introduction will describe the adhesions formed by keratinocytes, their regulation, and the mechanisms of signalling from such adhesions into the cell. The signalling processes taking place at these cell adhesions may involve members of the Src-family kinases and members of the protein kinase C family which will both be decribed in detail. In addition, the techniques used for the study of keratinocytes *in vitro* are described and the factors which can induce differentiation in these model systems are summarised.

CHAPTER 2

CELL ADHESION AND INTRACELLULAR SIGNALLING IN THE EPIDERMIS

2.1 CELL-MATRIX ADHESION

The structural integrity of the skin requires the physical attachment of the epidermis to the underlying dermis. This function is mediated by adhesive interactions between the basal keratinocytes and the adjacent basal lamina, a layer of proteinaceous material that includes laminins (Timpl *et al.,* 1979), collagens (Kefalides, 1975), kalinin (Rouselle *et al.,* 1991) and in wounded adult skin, fibronectin (Clark *et al.,* 1982). These extracellular matrix (ECM) proteins serve as ligands for the integrin-mediated adhesions of the basal keratinocytes, which tether the cells to the basement membrane. The integrins are a family of heterodimeric receptors comprising one transmembrane α-subunit and one transmembrane β-subunit and they bind specifically to different components of the extracellular matrix. This specificity is achieved by the existence of a variety of α- and β-subunits which can associate with one another in a range of different combinations. For example, the $\alpha_5\beta_1$ integrin binds most strongly to a tripeptide sequence in fibronectin (Pierschbacher and Ruoslahti, 1984), whereas the $\alpha_3\beta_1$ integrin binds to laminin 5 (Carter *et al.,* 1991). Keratinocytes form at least two types of cell-matrix adhesion involving integrins and these are likely to have different roles. In normal epidermis, the principal integrin-containing adhesion is the hemidesmosome although the focal adhesion may also be important under certain conditions. Integrin-mediated cell-matrix interactions are confined to the keratinocytes of the basal layer and the integrins are normally degraded early in the *in vivo* differentiation process (Peltonen *et al.,* 1989).

2.1.1 The hemidesmosome

Hemidesmosomes mediate adhesion between basal keratinocytes and laminin 5 in the basement membrane (Jones and Green, 1991; Sonnenberg *et al.,* 1993) and are the only type of integrin-containing adhesion to interact with the keratin intermediate filaments (Figure 2 *a*). The hemidesmosome utilises a single combination of integrins, namely the

α_6 and β_4 subunits (Sonnenberg *et al.*, 1991), both of which are restricted to the basal surface of basal keratinocytes in the epidermis (Carter *et al.*, 1990a) and preferentially associate with one another (Giancotti *et al.*, 1992). It has been suggested that the loss of the $\alpha_6\beta_4$ complex early in the keratinocyte differentiation process may facilitate the movement of the differentiating cell out of the basal layer (Tennenbaum *et al.*, 1996).

A. The structure of the hemidesmosome

The β_4 subunit differs from other β-integrin subunits in that it has a very large cytoplasmic domain of more than 1000 amino acid residues (Hogervorst *et al.*, 1990) which is involved in its indirect interaction with keratin filaments (Spinardi *et al.*, 1993). This interaction may occur *via* the 230kDa bullous pemphigoid antigen (BPAG1), which is localised to the region of hemidesmosomes where keratin filaments attach (Jones *et al.*, 1994). BPAG1 is a member of the plakin family of intermediate-filament (IF) binding proteins (Uitto *et al.*, 1996), which also includes desmoplakin, envoplakin and plectin. The carboxy-terminus of BPAG1 has homology with the IF-binding regions of the other family members and it is predicted that BPAG1 also binds to intermediate filaments (Green *et. al.*, 1992a). In addition, plectin may also bind to the cytoplasmic tail of the β_4-integrin (Borradori and Sonnenberg, 1996).

The 180kD bullous pemphigoid antigen (BPAG2) is also a component of hemidesmosomes and is a transmembrane protein with its amino terminus on the cytoplasmic side of the plasma membrane. BPAG2 may interact directly with the α_6 subunit as these two proteins coprecipitate irrespective of the presence of a β subunit (Hopkinson, 1995), and is likely to interact with the extracellular matrix *via* a series of collagen-like repeats in the extracellular domain (Giudice *et al.*, 1991). However, BPAG2 is not thought to directly bind to the intermediate filaments. Another component of hemidesmosomes is the 300kDa intermediate filament associated protein (IFAP), also present in desmosomes (Skalli *et al.*, 1994) which may be also involved in mediating interactions between the adhesion complex and the keratin intermediate filaments.

Figure 2

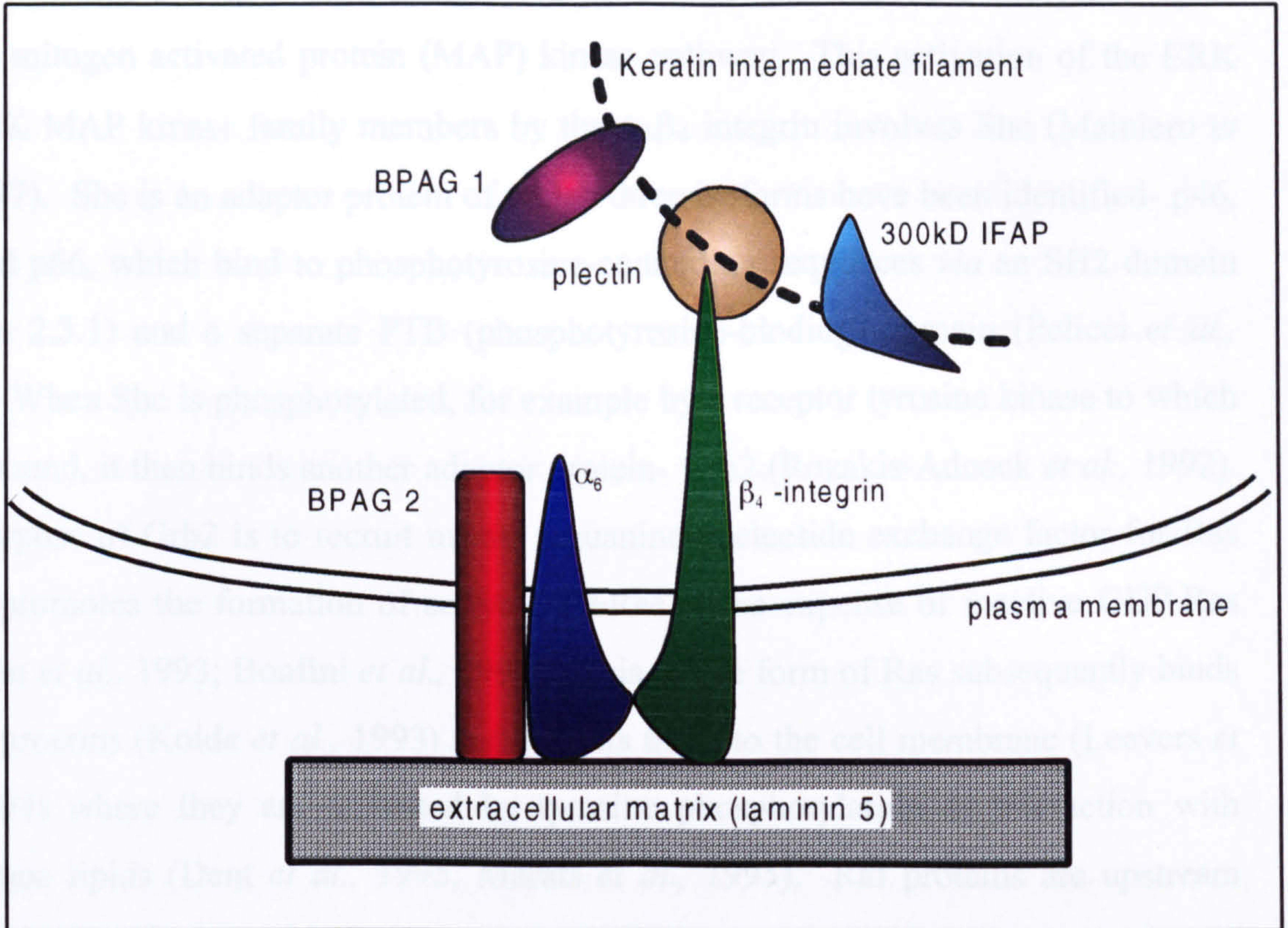
Figure 2
Cell-extracellular matrix adhesions formed by keratinocytes

a. The hemidesmosome.

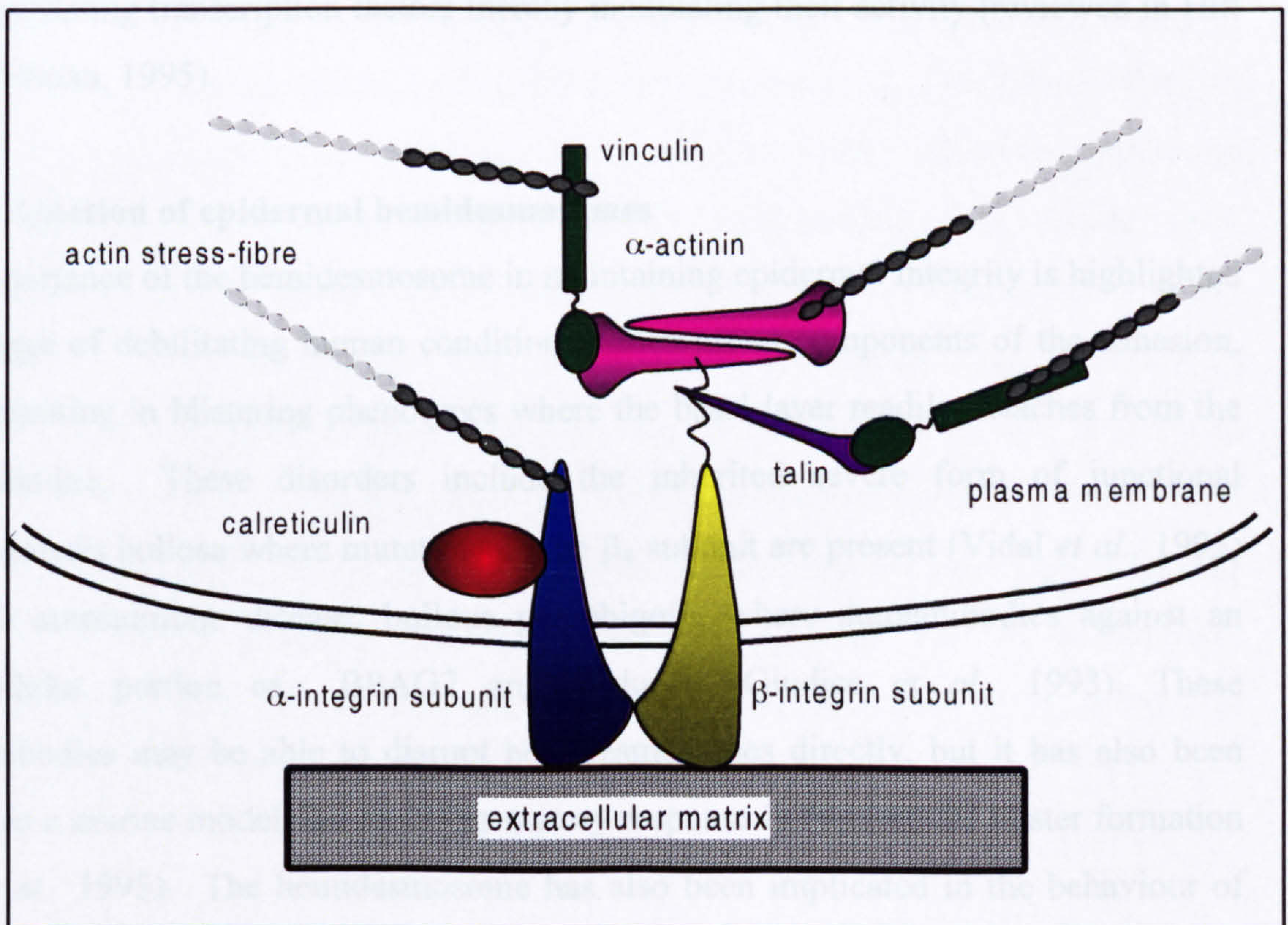
b. The focal adhesion. This diagram does not show the numerous signalling proteins that are also present in the focal adhesion complex.

Cell-extracellular matrix adhesions formed by keratinocytes

***a.* The hemidesmosome**



***b.* The focal adhesion**



B. Signalling from hemidesmosomes

In addition to playing an important mechanical function in maintaining the integrity of the epidermis, there is also evidence that hemidesmosomes transmit signals into the cell. Hemidesmosomal interaction with ligand has been shown to induce a mitogenic signal *via* the mitogen activated protein (MAP) kinase pathway. This activation of the ERK and JNK MAP kinase family members by the $\alpha_6\beta_4$ integrin involves Shc (Mainiero *et al.*, 1997). Shc is an adaptor protein of which three isoforms have been identified- p46, p52 and p66, which bind to phosphotyrosine-containing sequences *via* an SH2 domain (section 2.3.1) and a separate PTB (phosphotyrosine-binding) domain (Pelicci *et al.*, 1992). When Shc is phosphorylated, for example by a receptor tyrosine kinase to which it has bound, it then binds another adaptor protein- Grb2 (Rozakis-Adcock *et al.*, 1992). The purpose of Grb2 is to recruit mSos, a guanine-nucleotide exchange factor for Ras which promotes the formation of active GTP·Ras at the expense of inactive GDP·Ras (Chardin *et al.*, 1993; Bonfini *et al.*, 1992). This active form of Ras subsequently binds to Raf proteins (Koide *et al.*, 1993) and recruits them to the cell membrane (Leevers *et al.*, 1994) where they are activated by tyrosine phosphorylation or interaction with membrane lipids (Dent *et al.*, 1995; Marais *et al.*, 1995). Raf proteins are upstream activators of members of the mitogen-activated protein (MAP) kinase family of serine/threonine kinases (Lange-Carter *et al.*, 1993) which alter cell behaviour by phosphorylating transcription factors thereby modulating their activity (reviewed in Hill and Treisman, 1995).

C. The function of epidermal hemidesmosomes

The importance of the hemidesmosome in maintaining epidermal integrity is highlighted by a range of debilitating human conditions which affect components of the adhesion, often resulting in blistering phenotypes where the basal layer readily detaches from the basal lamina. These disorders include the inherited severe form of junctional epidermolysis bullosa where mutations in the β_4 subunit are present (Vidal *et al.*, 1995) and the autoimmune disease, bullous pemphigoid, where autoantibodies against an extracellular portion of BPAG2 are produced (Giudice *et al.*, 1993). These autoantibodies may be able to disrupt hemidesmosomes directly, but it has also been shown in a murine model that an inflammatory response is required for blister formation (Liu *et al.*, 1995). The hemidesmosome has also been implicated in the behaviour of

malignant cells where the expression of the $\alpha_6\beta_4$ integrin in the tumour parenchyma of squamous cell carcinoma was found to correlate with an increased rate of tumour recurrence after therapy (Wolf *et al.*, 1990).

2.1.2 The focal adhesion

The other form of integrin-mediated adhesion is the focal adhesion which, unlike the hemidesmosome, does not interact with keratin intermediate filaments (Figure 2 *b*), instead being bound to the actin cytoskeleton. The majority of studies on this type of adhesion have been performed in fibroblasts and although focal adhesions are formed by keratinocytes *in vitro* (Carter *et al.*, 1990a and b), it is not clear if they are formed by the keratinocytes of normal epidermis *in vivo*. However, focal adhesions are likely to play an important role in the epidermis when keratinocytes become motile, for example in the re-epithelialisation of wounded skin (Gates *et al.*, 1994). During such processes of tissue re-modelling, different integrins are temporarily expressed in the epidermis, including the fibronectin receptor $\alpha_5\beta_1$ which is required for migration over provisional fibronectin-containing matrices (Cavani *et al.*, 1993).

Unlike the hemidesmosome, this type of cell-extracellular matrix (ECM) adhesion can involve a variety of integrin heterodimers with the exception of the hemidesmosomal $\alpha_6\beta_4$ integrin. The principal non-hemidesmosomal keratinocyte integrins in normal adult epidermis are the $\alpha_3\beta_1$ laminin 5 receptor and the $\alpha_2\beta_1$ collagen/laminin receptor (Kramer and Marks, 1989; Carter *et al.*, 1991), both of which are normally restricted to the basal layer (Peltonen *et al.*, 1989). However, unlike the $\alpha_6\beta_4$ receptor, these integrins are not restricted to the basal surface of the basal cells and are also present on their lateral surfaces where the normal extracellular ligands may not be present. There is some evidence that the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in this location may contribute to cell-cell adhesion and it has been demonstrated that the $\alpha_2\beta_1$ integrin can interact directly with $\alpha_3\beta_1$ (Symington *et al.*, 1993), possibly influencing differentiation (Symington and Carter, 1995). However, studies by other authors have argued that the laterally distributed integrins play no significant physical role in keratinocyte intercellular adhesion except under conditions where the formation of other types of cell-cell adhesion is suppressed (Tenchini *et al.*, 1993).

A. The structure of the focal adhesion

The focal adhesion transmits force into the cell *via* the actin cytoskeleton and a number of important adhesion components are involved in mediating interactions between the integrins and the actin (Figure 2 *b*). In fibroblasts, the focal adhesions are at the termini of the actin stress fibres and although the actin cytoskeleta of keratinocytes are arranged differently from those of fibroblasts, a similar relationship is likely to exist, at least *in vitro* (Kubler *et al.*, 1991). In addition, the focal adhesion has a marked influence on the organisation of the cytoskeleton, particularly in fibroblasts where the formation of adhesions causes the reorganisation of the actin filaments (Badley *et al.*, 1980).

The intracellular carboxy-terminal five amino acids of α_2-integrin can interact directly with filamentous (F)-actin (Kieffer *et al.*, 1995). In addition, the β_1-subunit has been shown to interact with actin-binding proteins such as α-actinin (Otey *et al.*, 1990) and talin (Horwitz *et al.*, 1986), which can directly bridge the β_1-subunit to F-actin. There are also other proteins present in the focal adhesion that serve to tether the integrins to the actin cytoskeleton in a more indirect manner including the actin-binding protein vinculin (Jockusch and Isenberg, 1981), which binds to both talin and α-actinin (Jones *et al.*, 1989; McGregor *et al.*, 1994).

B. Signalling to and from the focal adhesion

In addition to performing an adhesive role, focal adhesions act as important mediators of cellular signalling, which can be broadly divided into two types- 'inside-out' and 'outside-in'.

Inside-out signalling

Inside-out signalling is the phenomenon whereby the affinity of the integrin for its ligand is regulated from within the cell. This is not fully understood but may involve conformational changes in the integrin subunits (Puzon-McLauglin *et al.*, 1996). A potential mediator of this process is the Ca^{++}-binding protein calreticulin which directly binds to the α_2 subunit and may serve to stabilise the high affinity state of the $\alpha_2\beta_1$ receptor (Coppolino *et al.*, 1995; Michalak, 1992). More recently it was demonstrated that the presence of calreticulin is essential for integrin-mediated cell-matrix adhesion

(Coppolino *et al.,* 1997), confirming that this protein has an important role in the control of focal adhesions.

Outside-in signalling

Focal adhesions also transduce signals into the cell, with the result that attachment to extracellular matrix profoundly influences the behaviour of the cell. When many normal epithelial cell types are deprived of all integrin contacts or are in contact with extracellular matrix of inappropriate composition, they undergo programmed cell death, a phenomenon that has been termed 'anoikis' (Frisch and Francis, 1994). This integrin-mediated selective survival may have evolved to prevent cells from growing in an inappropriate location where they would be isolated from their normal regulatory influences and could have a deleterious effect. In many malignancies, tumorigenic cells either show altered integrin expression or have circumvented their requirement for matrix attachment altogether (reviewed in Ziober *et al.,* 1996). Signalling through focal adhesions is a complex process involving more than one signalling pathway. When integrins engage extracellular matrix ligands, an increase in the tyrosine phosphorylation of a number of proteins is observed (Juliano and Haskill, 1993) and serine/threonine kinases such as protein kinase C (PKC) are also activated (Vuori and Ruoslahti, 1993). An important tyrosine kinase in focal adhesion-mediated signalling is the 125kD focal adhesion kinase ($pp125^{FAK}$), which binds to β_1-integrin and becomes autophosphorylated upon integrin engagement (Schaller *et al.,* 1994). A number of proteins of known signalling function interact with $pp125^{FAK}$ in an integrin-engagement dependent manner, including Grb2, mSos1 and members of the Src-family kinases (Schlaepfer *et al.,* 1994). The binding of the adapter protein Grb2 to $pp125^{FAK}$ recruits mSos leading to Ras and subsequently MAP kinase activation, a similar phenomenon to that described for hemidesmosomes in section 2.1.1C (Chen *et al.,* 1994; Schlaepfer *et al.,* 1994).

C. The role of epidermal focal adhesions

The importance of $pp125^{FAK}$ in keratinocytes *in vivo* is unclear as this protein is undetectable in normal adult epidermis but is expressed during wound healing (Gates *et al.,* 1994), and by keratinocytes propagated *in vitro* (Xia *et al.,* 1996). This suggests that

the non-hemidesmosomal integrin-mediated cell adhesions formed in normal intact epidermis may not represent true focal adhesions.

2.1.3 Integrins and keratinocyte behaviour

Abnormalities in integrin expression levels and patterns in psoriasis and epidermal neoplasms suggest that the integrins may exert a direct influence on keratinocyte proliferation and differentiation. In psoriasis, a benign disease of the epidermis where suprabasal keratinocyte hyperproliferation occurs (Weinstein and Van Scott, 1965) and differentiation is disturbed (Bernard *et al.,* 1988), integrins are present in the suprabasal layers. In addition, integrin heterodimers that are not normally present in adult human epidermis, such as $\alpha_5\beta_1$, are expressed (Pellegrini *et al.,* 1992). Compelling evidence for a direct link between suprabasal integrin expression and keratinocyte hyperproliferation was obtained by the enforced expression of the β_1-integrin subunit in the suprabasal layers of murine epidermis, resulting in a phenotype closely resembling psoriasis (Carroll *et al.,* 1995). In addition, studies of epidermal neoplasms have shown that the $\alpha_6\beta_4$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are expressed suprabasally in some benign squamous papillomas and more homogenously in primary and metastatic squamous cell carcinomas (Van Waes *et al.,* 1995).

A number of *in vitro* studies have also demonstrated the importance of integrins in the control of human keratinocyte differentiation. The observation, made by Green in 1977, that depriving keratinocytes of matrix contact (section 3.2.4) is sufficient to induce aspects of the differentiation process has now been extended considerably. Separation from the matrix induces an irreversible cessation of DNA synthesis and the induction of involucrin expression (Watt *et al.,* 1988). Soon after cells are separated from the extracellular matrix, the integrins present on the cell surface lose their adhesive function and the synthesis of new integrins is inhibited (Hotchin *et al.,* 1995; Nicholson and Watt, 1991; Adams and Watt, 1990). At later times, when the cells have committed to the differentiation process and are synthesising involucrin, the cell-surface integrins are internalised and degraded (Hotchin *et al.,* 1995; Watt *et al.,* 1988). Confirmation of the importance of one integrin heterodimer in this process came when it was shown that the presence of soluble fibronectin suppressed the induction of involucrin expression in keratinocytes deprived of matrix contact, directly implicating the $\alpha_5\beta_1$ fibronectin

receptor in the control of the differentiation program (Adams and Watt, 1989). Furthermore, involucrin induction is also suppressed by an antibody which blocks β_1-integrin function (Watt *et al.,* 1993). Indeed, other ligands of β_1-containing heterodimers were subsequently implicated in the control of differentiation when it was shown that the addition of laminin and collagen IV in the presence of small amounts of fibronectin also inhibits suspension-induced differentiation (Watt *et al.,* 1993). Other studies have suggested a role for the $\alpha_3\beta_1$ laminin 5 receptor in the modulation of differentiation, on the basis that antibodies which promote cell-cell adhesion (and inhibit cell-matrix adhesion) mediated by this heterodimer can induce some aspects of differentiation (Symington and Carter 1995). These studies on the control of keratinocyte differentiation by the integrins have led to suggestions that detachment from the basement membrane may play a crucial role in the induction of differentiation *in vivo.* In this model (described in Hotchin *et al.,* 1993), if a basal keratinocyte does not form integrin-mediated interactions with the extracellular matrix for a certain period of time, conformational changes in the integrin receptors occur and the cell commits to differentiation. The consequence of the altered integrin conformation is a reduction in the ability of the cell to bind extracellular matrix resulting in the displacement of the cell from the basal layer of the epidermis. This cell now internalises and degrades the cell-surface integrins whilst differentiating further and moving towards the surface of the skin.

2.2 CELL-CELL ADHESION

The integrity of the epidermis is maintained by the inter-keratinocyte adhesions present throughout the tissue. In the cornified layer, the cells are held together by the interdigitations of their extremities and perhaps also by molecular interactions between their specialised lipid envelopes (Wertz *et al.,* 1989). Deeper in the epidermis, more complex interactions are found, in the form of cadherin-mediated adherens junctions and desmosomes. Both of these two main types of keratinocyte intercellular adhesion are Ca^{++}-dependent and utilise members of the cadherin family to mediate their adhesive interactions. However, the classical cadherins of the adherens junction interact indirectly with the actin cytoskeleton whereas the desmosomal cadherins indirectly

interact with the keratin intermediate filaments. As its name suggests, the desmosome has many properties and components in common with the hemidesmosome (section 2.1.1).

2.2.1 The desmosome

Desmosomes (Figure 3 *a*) are present in all the living layers of the epidermis. During the differentiation process they increase in both number and size and use different isoforms of some of their components (Arnemann *et al.*, 1993; Skerrow *et al.*, 1989; Chapman and Walsh, 1990) before being disassembled during the transition between the upper granular layer and the cornified layer (King *et al.*, 1987). However, some desmosomal components persist in the cornified layer and are incorporated into the structure of the cornified envelope, raising the possibility that the keratin intermediate filaments could be attached to the envelope *via* these desmosomal remnants (Robinson *et al.*, 1997).

A. The structure of desmosomes and their association with intermediate filaments

The transmembrane desmosomal glycoproteins that are involved in cell-cell adhesion are the desmogleins and the desmocollins, both having partial homology to the classical cadherins in their extracellular domains (Goodwin *et al.*, 1990; Holton *et al.*, 1990). The way in which these proteins mediate cell-cell adhesion is not fully understood but it is possible that a heterodimer containing a desmoglein and a desmocollin may comprise the basic adhesive structure (Chitaev and Troyanovsky, 1997). There are three desmoglein isoforms encoded by different genes, Dsg1, Dsg2 and Dsg3 (Buxton *et al.*, 1993), all of which contain a binding site for the catenin, plakoglobin (section 2.2.2B) in the cytoplasmic tail. There are also three desmocollin genes, *dsc*1, *dsc*2 and *dsc*3 (King *et al.*, 1995), each of which gives rise to two alternatively spliced mRNA transcripts encoding an 'a' form and a 'b' form. The a-forms contain an additional 11 amino acids in the cytoplasmic domain, including a plakoglobin binding site which is absent from the b-forms (Troyanovsky *et al.*, 1994a). The desmocollins and desmogleins associate indirectly with the keratin intermediate filaments *via* proteins that include desmoplakin, plakoglobin and possibly IFAP 300 (Skalli *et al.*, 1994).

Figure 3

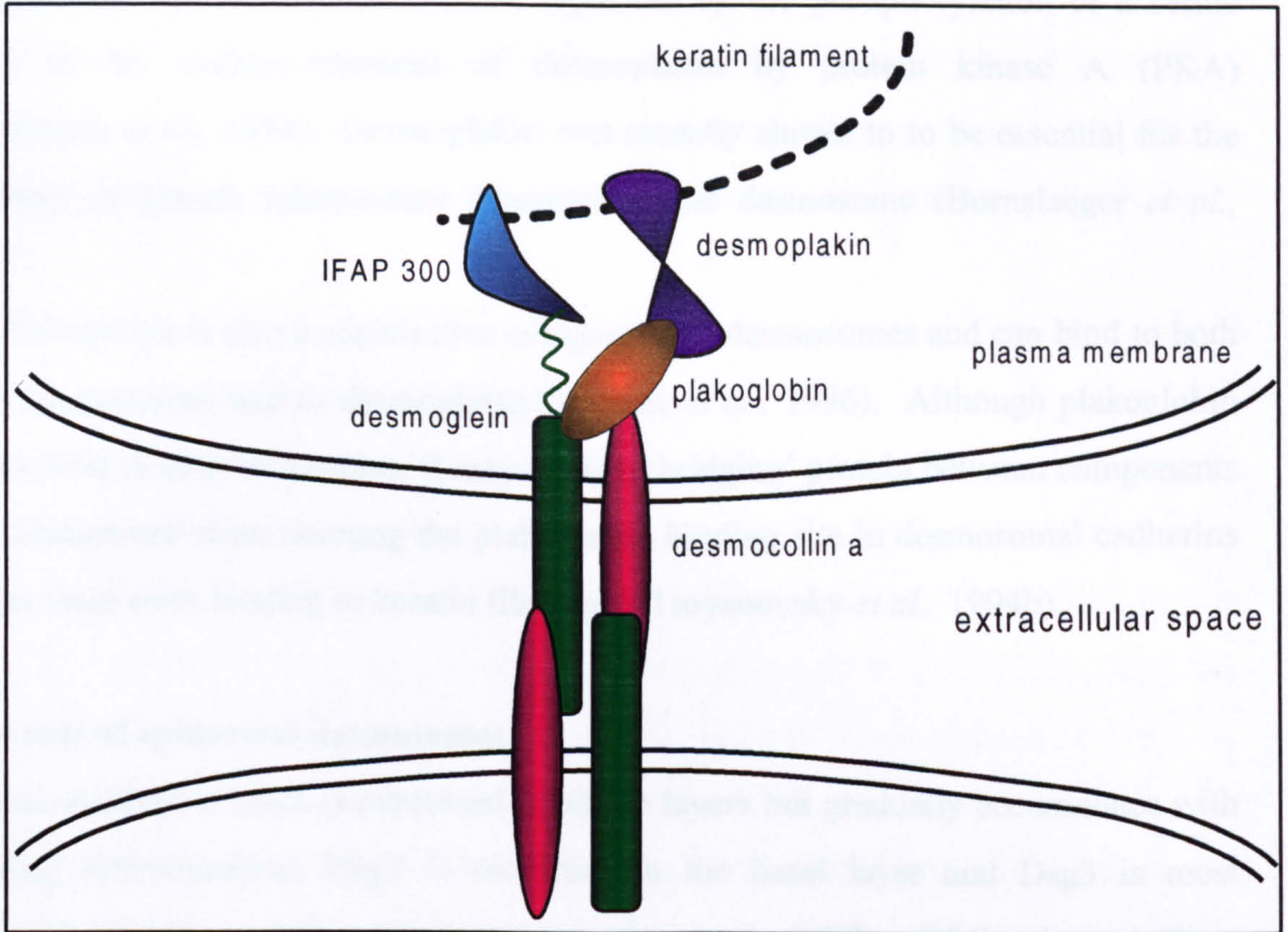
Figure 3
Cell-cell adhesions formed by keratinocytes

a. The desmosome.

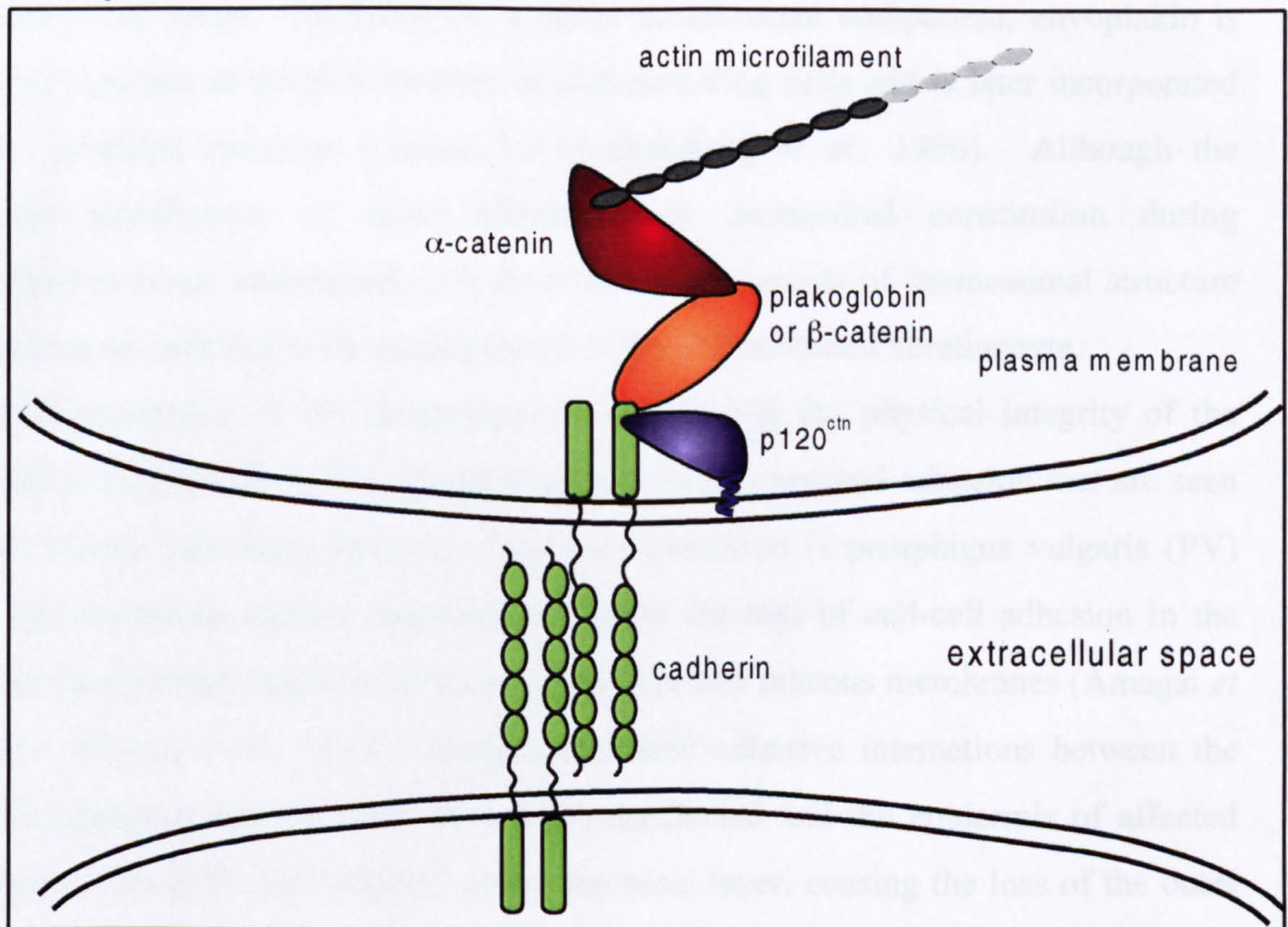
b. The cadherin-mediated adherens junction.

Cell-cell adhesions formed by keratinocytes

***a.* The desmosome**



***b.* The adherens junction**



Desmoplakin is a dumbell-shaped molecule with a central α-helical coiled rod domain flanked by two globular end domains (Green *et al.,* 1992b) and the carboxy-terminus interacts directly with type II epidermal keratins including K1 and K5 (Kouklis *et al.,* 1994). This association may be regulated by the phosphorylation of a serine residue in the carboxy-terminus of desmoplakin by protein kinase A (PKA) (Stappenbeck *et al.,* 1994). Desmoplakin was recently shown to to be essential for the attachment of keratin intermediate filaments to the desmosome (Bornslaeger *et al.,* 1996).

Plakoglobin is also a constitutive component of desmosomes and can bind to both a-form desmocollins and to desmogleins (Witcher *et al.,* 1996). Although plakoglobin does not bind directly to keratins, it may act as a 'bridging' protein between components of the desmosome since deleting the plakoglobin binding site in desmosomal cadherins prevents them from binding to keratin filaments (Troyanovsky *et al.,* 1994b).

C. The role of epidermal desmosomes

In human epidermis, Dsg1 is expressed in all the layers but gradually accumulates with increasing differentiation, Dsg2 is restricted to the basal layer and Dsg3 is most prevalent in the lower spinous layers (Arnemann *et al.,* 1993). Of the desmocollins, Dsc1 is restricted to the upper layers of the epidermis whereas Dsc2 and Dsc3 are expressed in all layers. Furthermore, a novel desmosomal component, envoplakin is exclusively present in the desmosomes of differentiating cells and is later incorporated into the cornified envelope (section 1.2.1) (Ruhrberg *et al.,* 1996). Although the functional significance of these alterations in desmosmal constitution during differentiation is not understood, it is clear that some aspects of desmosomal structure and function are tailored to the requirements of the differentiated keratinocyte.

The importance of the desmosome in maintaining the physical integrity of the epidermis is highlighted by the effects of defects in desmosomal adhesion that are seen in some human blistering diseases. One such condition is pemphigus vulgaris (PV) where autoantibodies against desmoglein 3 cause the loss of cell-cell adhesion in the basal and immediately suprabasal layers of the skin and mucous membranes (Amagai *et al.,* 1991; Buxton *et al.,* 1993). Integrin-mediated adhesive interactions between the basal keratinocytes and the basal lamina are unaffected and the epidermis of affected individuals often splits immediately above the basal layer, causing the loss of the outer

layers of cells leaving large eroded areas susceptible to infection and water loss. Without treatment, this condition is almost invariably fatal (Stanley, 1993). There has been some debate as to the role of the Dsg3 autoantibodies in this condition and it has been suggested that blister formation requires the release of proteases from affected keratinocytes (Morioka *et al.,* 1987). However, the recent production of a Dsg3 -/- mouse has shown that the absence of this desmoglein is sufficient to cause a blistering phenotype which closely resembles, but is not identical to, pemphigus vulgaris (Koch *et al.,* 1997).

2.2.2 The cadherin-mediated adherens junction

Cell-cell adhesions of this type (Figure 3 *b*) involve the classical cadherins (including E-, P- and N-cadherin) and require the presence of calcium ions (Ca^{++}) for their formation and perpetuation (Takeichi, 1977). The adhesive function of the classical cadherins is destroyed by trypsin in the absence, but not in the presence, of Ca^{++} (Takeichi *et al.,* 1981), a feature that was used in the initial identification of some of these proteins. A number of cadherins have now been identified, including E- (epithelial), P- (placental), N- (neural), Ksp- (kidney specific, Thomson *et al.,* 1995), OB- (osteoblast, Okazaki *et al.,* 1994), LI- (liver/intestine, Berndorff *et al.,* 1994), M- (muscle, Donalies *et al.,*1991) and T-cadherin (truncated, Ranscht and Dours-Zimmermann, 1991). Some of these molecules are not considered classical cadherins, for example T-cadherin, which may not be involved in mediating cell-cell adhesion directly but may be important in cellular recognition (Koller and Ranscht, 1996). Human keratinocytes express two classical cadherins- E-cadherin and P-cadherin. Cadherins generally only form homophilic adhesions with identical cadherins on opposing cells, providing a mechanism that leads to the aggregation of 'like' cells and the exclusion of 'unlike' cells (Nose *et al.,* 1988), an important feature of tissue morphogenesis. This selectivity, in conjunction with the restricted tissue distribution of the cadherins means that these molecules play an important role in development. This is demonstrated by the E-cadherin nullizygous mice which fail to complete embryogenesis (Larue *et al.,* 1994).

A. Cadherin structure and function

E-cadherin is the best characterised of all the cadherins and its structure will now be examined in more detail. Much of this description is also applicable to P-cadherin, the other cadherin present in keratinocytes which shows extensive homology with E-cadherin. E-cadherin is a single pass transmembrane glycoprotein of 120kD which is derived from a larger 135kD precursor (Peyrieras *et al.,* 1993) by proteolysis and is subsequently phosphorylated (Vestweber and Kemler, 1984) and glycosylated (Hyafil *et al.,* 1980) during transport to the cell surface. The proteolysis of the precursor form to the smaller mature form is essential for the adhesive function of the protein and takes place before the protein is inserted into the cell membrane. Thus, the uncleaved form of E-cadherin is not normally inserted into the cell membrane. However, if the proteolytic site is mutated, the 135kD precursor can then be detected in the membrane, indicating that proteolytic cleaveage is not a pre-requisite for membrane insertion (Ozawa and Kemler, 1990). When the mature E-cadherin is inserted into the plasma membrane, the N-terminus is extracellular (Figure 4) and comprises five, 7-stranded β-barrel extracellular repeats (EC1-5) connected by 10-residue linkers. In between each pair of EC repeats is a pocket that binds three Ca^{++} held in a contiguous arrangement by negatively charged amino acid side-groups (Nagar *et al.,* 1996 and Overduin *et al.,* 1995). There are thus four potential Ca^{++}-binding pockets in the extracellular portion of each cadherin molecule, located at the flexible 'nodes' of the molecule. This provides a potential explanation for the globular shape of E-cadherin in the absence of Ca^{++}, and its rod shape in the presence of Ca^{++} (Pokutta *et al.,* 1994). In the presence of Ca^{++}, E-cadherin not only changes into a rigid rod shape but also forms dimers between parallel molecules projecting from the surface of the same cell, an interaction involving the N-terminal EC domains (Nagar *et al.,* 1996). In addition to the gross conformational changes induced in the presence of Ca^{++}, another part of the E-cadherin molecule that responds to Ca^{++} is the His^{79}-Ala-Val sequence in EC-1 (Overduin *et al.,* 1995; the numbering applies to murine E-cadherin), also present in P-cadherin and N-cadherin. This tripeptide sequence is thought to be the site where the initial homophilic interactions between cadherin molecules are made (Blaschuk *et al.,* 1990). These interactions are believed to occur between the HAV sequence and a membrane-proximal region immediately preceding the transmembrane domain of the cadherin projecting

from an adjacent cell, a site where adhesion-disrupting antibodies interact (Ozawa *et al.*, 1990; Hatta *et al.*, 1988).

B. Interactions with the cytoskeleton

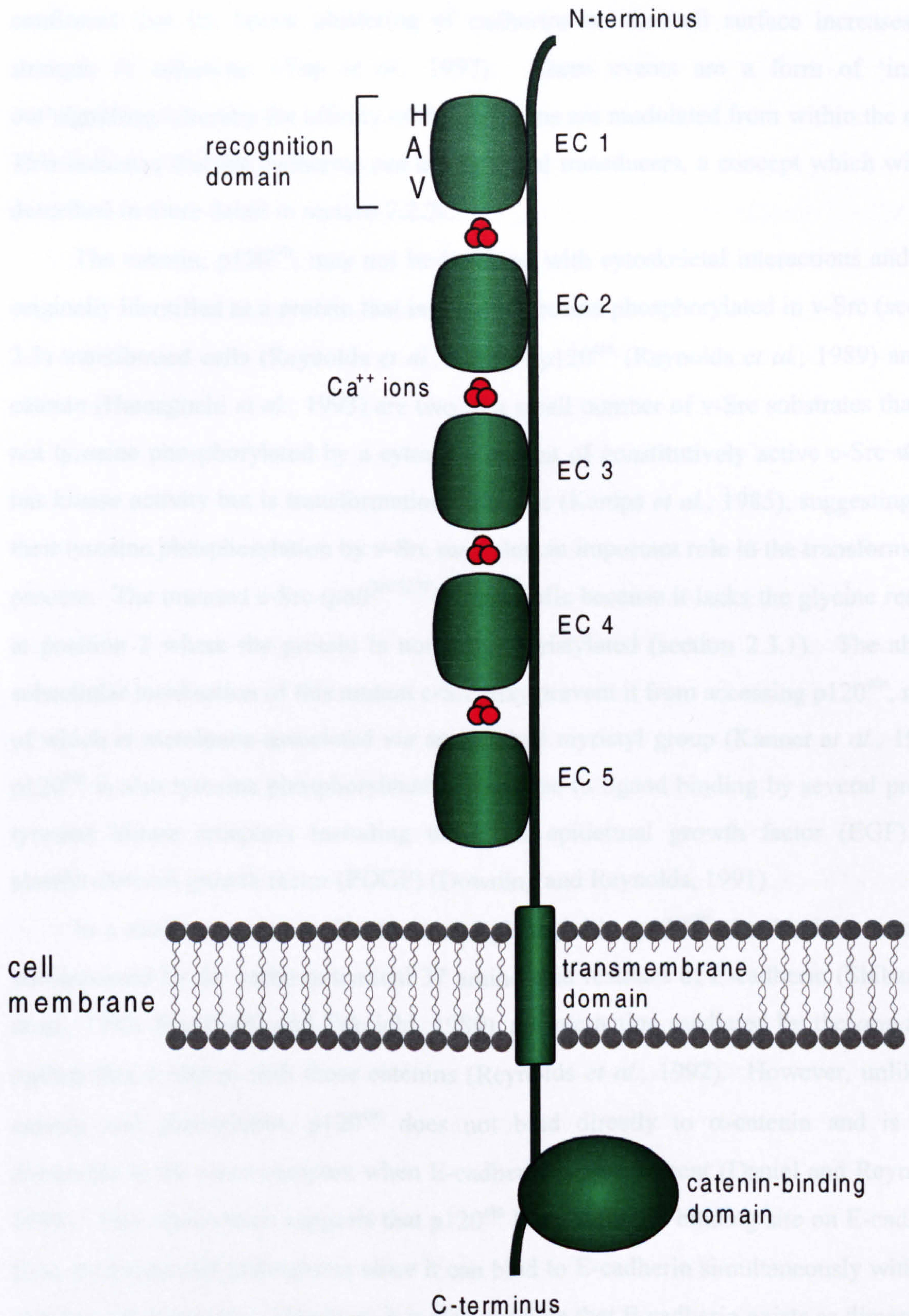
The classical cadherins are indirectly associated with the actin cytoskeleton by proteins of the catenin family which bind to the immature cadherin before it is inserted into the cell membrane (Figure 3 *b* ;Ozawa *et al.*, 1989; Ozawa and Kemler, 1992). At least six catenins have been identified in mammalian cells, including α-catenin, β-catenin, plakoglobin (or γ-catenin), $p120^{ctn}$ (previously named $p120^{cas}$, Reynolds and Daniel, 1997) and recently p0071 (Hatzfeld and Nachtsheim, 1996) and ARV (Sirotkin *et al.*, 1997), of which α-catenin and either β-catenin or plakoglobin are required to mediate interactions with the actin cytoskeleton. β-catenin, plakoglobin, $p120^{ctn}$, p0071 and ARV all have homology with the *Drosophila* segment polarity-determining protein Armadillo and contain a number of the *armadillo* protein-protein interaction motifs originally identified in this protein (Riggleman *et al.*, 1989). Either β-catenin (94kD) or plakoglobin (83kD) binds directly to a 25-amino acid domain in the cytoplasmic portion of the cadherin molecule (Jou *et al.*, 1995), although plakoglobin is also involved in the formation of desmosomes (section 2.2.1), and is preferentially associated with this type of adhesion (Peifer *et al.*, 1992; Nathke *et al.*, 1994). Neither plakoglobin nor β-catenin has any actin-binding function and must interact with an additional member of the catenin family to indirectly tether cadherins to the cytoskeleton. This actin-binding function is performed by a single catenin, namely α-catenin, a 102kD protein with homology to vinculin (but not Armadillo) that directly binds and can also bundle actin filaments (Rimm *et al.*, 1995; Nagafuchi *et al.*, 1991). α-catenin can bind to both β-catenin and plakoglobin, but binds to β-catenin more strongly (Obama and Ozawa, 1997). In addition, α-catenin can oligomerise with itself, or with the actin-binding proteins vinculin and α-actinin (Knudsen *et al.*, 1995), serving to cluster cadherins on the surface of the cell, possibly producing a high affinity site for cell-cell interaction (Ozawa *et al.*, 1989).

Figure 4

Figure 4
The structure of E-cadherin

A schematic representation of the E-cadherin protein. The extracellular portion of mature E-cadherin contains five repeats (EC1-5) which are predicted to be β-barrel structures. Between each EC repeat is a calcium-binding pocket, formed by the adjacent EC repeats, each of which binds three Ca^{++} ions, stabilised by negatively-charged amino acid side-chains that project into the binding pocket. In the first, N-terminal repeat is the HAV motif which is conserved between many cadherins and is believed to form the recognition domain of the molecule, possibly interacting with a region between EC5 and the cell membrane of opposing cadherin molecules on adjacent cells. The intracellular portion of the protein contains the catenin binding domain, in the C-terminal 25 amino acids.

The structure of E-cadherin



Indeed, it has been suggested that the formation of homophilic adhesions could be controlled by this lateral movement of cadherins on the cell surface since the formation of parallel dimers between adjacent E-cadherin molecules *via* the EC-1 repeats precedes homophilic interaction *in vitro* (Tomschy *et al.,* 1996). More recent studies have confirmed that the lateral clustering of cadherins on the cell surface increases the strength of adhesions (Yap *et al.,* 1997). These events are a form of 'inside-out' signalling whereby the affinity of the cadherins are modulated from within the cells. This indicates that the cadherins can act as signal transducers, a concept which will be described in more detail in section 2.2.2C.

The catenin, $p120^{ctn}$, may not be involved with cytoskeletal interactions and was originally identified as a protein that is heavily tyrosine phosphorylated in v-Src (section 2.3) transformed cells (Reynolds *et al.,* 1989). $p120^{ctn}$ (Reynolds *et al.,* 1989) and β-catenin (Hamaguchi *et al.,* 1993) are two of a small number of v-Src substrates that are not tyrosine phosphorylated by a cytosolic mutant of constitutively active c-Src which has kinase activity but is transformation-defective (Kamps *et al.,* 1985), suggesting that their tyrosine phosphorylation by v-Src may play an important role in the transformation process. The mutated c-Src ($p60^{2A/527F}$) is cytosolic because it lacks the glycine residue at position 2 where the protein is normally myristylated (section 2.3.1). The altered subcellular localisation of this mutant c-Src may prevent it from accessing $p120^{ctn}$, much of which is membrane-associated *via* an attached myristyl group (Kanner *et al.,* 1991). $p120^{ctn}$ is also tyrosine phosphorylated in response to ligand binding by several protein tyrosine kinase receptors including those for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Downing and Reynolds, 1991).

In a similar manner to β-catenin and plakoglobin, $p120^{ctn}$ also binds to a region encompassed by the carboxy-teminal 37 amino acid residues of E-cadherin (Shibamoto *et al.,* 1995; Nagafuchi and Takeichi, 1989), an interaction mediated by the *armadillo* repeats that it shares with these catenins (Reynolds *et al.,* 1992). However, unlike β-catenin and plakoglobin, $p120^{ctn}$ does not bind directly to α-catenin and is only detectable in the same complex when E-cadherin is also present (Daniel and Reynolds, 1995). This observation suggests that $p120^{ctn}$ has a different binding site on E-cadherin from β-catenin and plakoglobin since it can bind to E-cadherin simultaneously with one of these other catenins. However, it is also possible that E-cadherin exists as dimers and

that p120^{ctn} is bound to one E-cadherin molecule whilst the other E-cadherin molecule binds either plakoglobin or β-catenin.

The messenger RNA (mRNA) for p120^{ctn} is present in a wide variety of tissues, suggesting that the protein is also widely expressed (Reynolds *et al.*, 1992). It is now known that a number of p120^{ctn} isoforms exist, with different tissue distributions, implying that they may have diverse functions in different tissues (Keirsebilck and van Roy, 1997). These first four isoforms to be described were grouped into two pairs on the basis of their size, the nomenclature of which was based on the previous name for p120^{ctn} (p120^{cas})- CAS1A and CAS1B are approximately 120kD whereas CAS2A and CAS2B are 100kD is size. In general CAS1 isoforms (now referred to as p120^{ctn}1)are expressed in mesenchymal cells such as fibroblasts and CAS2 isoforms (now referred to as p120^{ctn}3) are predominantly expressed in epithelial cells. Intriguingly, a variety of carcinoma cell lines show aberrant expression of the CAS1 isoforms and some lose the CAS2 isoforms that would normally be present (Mo and Reynolds, 1996) perhaps indicating a loss of the epithelial phenotype. All four isoforms are derived by variations in the mRNA splicing process and are predicted to interact with cadherins since they all contain the *armadillo* repeats that are known to be sufficient for association with E-cadherin (Daniel and Reynolds, 1995; Staddon *et al.*, 1995). However, there are likely to be functional differences between the p120^{ctn} isoforms. For example, the largest p120^{ctn} isoforms (p120^{ctn}1) are associated with the cytosolic protein tyrosine kinase Fer, whereas the shorter isoforms are not (Kim and Wong, 1995). Fer is a non-Src-family kinase which has a single SH2 domain and a large amino terminal domain of unknown function (Hao *et al.*, 1989). The functional significance of this association is not known but may not be important in keratinocytes which do not express the p120^{ctn}1 isoforms (section 9.2.3C). The cellular role of p120^{ctn} is not yet understood but there have been suggestions that it could interfere with the binding of β-catenin and plakoglobin to E-cadherin in transformed cells, thus disrupting cell-cell adhesion (Kinch *et al.*, 1995).

p120^{ctn} is the closest relative of the most recently described, ubiquitously expressed catenin which also contains *armadillo* repeats, ARV (armadillo repeat gene deleted in VCFS), which is defective in a significant proportion of patients suffering from velo-cardio-facial syndrome (VCFS). This disease is manifested as a variety of developmental abnormalities including heart defects, cleft palate and facial dysmorphology. Although a role for this novel catenin has yet to be demostrated, the

gene encoding ARV is hemizygous in a significant proportion of these VCFS patients (Sirotkin *et al.*, 1997), suggesting that ARV may play an important role in normal human development and morphogenesis.

Another novel catenin which is closely related to p120^{ctn} is p0071, again having a central *armadillo* repeat region. This ubiquitously expressed protein is localised to cadherin-mediated cell-cell adhesions and may also be a desmosomal component, suggesting that it may play some role in intercellular adhesion (Hatzfeld and Nachtstein, 1996).

C. Signalling to and from cadherin-mediated adherens junctions

In a similar manner to some other types of cell adhesion, adherens junctions can act as two-way signal transducing structures with signals from within the cell acting to regulate the stability of the junction or the junction modulating the behaviour of the cell.

Inside-out signalling

The regulation of this type of adhesion from within the cell is beginning to be elucidated. For example, in 1995 Takeda and co-workers showed that increased v-Src tyrosine kinase activity caused cadherin-mediated epithelial cell-cell adhesions to alter from a strong state to a weaker state, suggesting that tyrosine phosphorylation can influence junction stability. This destabilisation is concurrent with increased tyrosine phosphorylation of β-catenin (Matsuyoshi *et al.*, 1992) although β-catenin is not required for the v-Src-induced weakening of these cell-cell adhesions (Takeda *et al.* 1995). Another junction component, the actin-capping protein radixin (Tsukita *et al.*, 1989) is also tyrosine phosphorylated in v-Src transformed epithelial cells (Takeda *et al.*, 1995), potentially disrupting interactions between the adhesions and the actin cytoskeleton.

Serine/threonine phosphorylation may also play a role in the control of these adhesions. Inactivating Ser/Thr phosphatases with okadaic acid or calyculin A causes the disruption of cadherin-mediated cell-cell adhesions (Serres *et al.*, 1997) and decreasing Ser/Thr phosphorylation using the protein kinase inhibitor H-7 prevents cells from breaking their adherens junctions upon withdrawal of extracellular Ca^{++} (Citi, 1992). Thus, reducing the Ser/Thr phosphorylation of cell-cell adhesion components appears to increase the stability of the junction. However, activating protein kinase C

(PKC) in low extracellular Ca^{++} is sufficient to induce the transient relocalisation of E-cadherin, α-catenin, β-catenin, α-actinin and vinculin, but not desmosomal components, to regions of cell-cell contact of keratinocytes propagated *in vitro* (Lewis *et al.,* 1994a). This response is similar to that induced by high extracellular Ca^{++}. In a complementary series of experiments, the same authors showed that depleting cellular levels of PKC can block the Ca^{++}-induced formation of cadherin-mediated adherens junctions. It is noteworthy that activating PKC also induces many features of keratinocyte differentiation *in vitro*, indicating that the formation of adherens junctions could be a component of this process (section 3.2.1; Parkinson and Emmerson, 1982). Thus, the relationship between phosphorylation and the stability of cadherin-mediated cell-cell adhesions is complex although it is clear that both tyrosine and serine/threonine phosphorylation can modulate this type of intercellular adhesion.

Outside-in signalling

The influence that can be exerted by the presence of cadherin-mediated cell-cell adhesions on cell behaviour indicates that these adhesions have functions beyond mediating homophilic physical interactions between cells. Signalling into the cells from this type of adhesion is relatively uncharacterised although a number of molecules with potential signalling function are now known to be components of these adhesions.

Three members of the Src-family kinases (section 2.3) colocalise with the cadherins in cell-cell junctions (Tsukita *et al.,*1991) although they have yet to be shown to physically associate with components of the adhesion. In addition, several different tyrosine phosphatases have been detected in association with the junctional complex including RPTPμ, a receptor protein tyrosine phosphatase (Brady-Kalnay *et al.,* 1995); a member of the LAR-PTP family, a leukocyte antigen-related transmembrane tyrosine phosphatase that dephosphorylates β-catenin (Kypta *et al.,* 1996); PCP-2, a receptor protein tyrosine phosphatase (Wang et al., 1996) and a PTP-1B-like phosphatase (Balsamo *et al.,* 1996). However, none of these molecules has been shown directly to mediate signalling from the adhesion and could be equally important in regulating junction stability as described above. A molecule that associates directly with the cadherins and could play an important role in signalling is the adapter protein Shc which may provide a mitogenic signal *via* Ras activation (see also section 2.1.1B), but again could also modulate junction stability (Xu *et al.,* 1997a).

D. Cadherins and the control of epithelial cell behaviour

In the epidermis, E-cadherin is expressed throughout the living layers whereas P-cadherin is normally restricted to the basal layer, being degraded as the keratinocytes enter the spinous layer (Fujita *et al.,*1992). There is now evidence that the formation of cadherin-mediated adhesions in the epidermis and in other epithelial tissues plays an important role in the control of cellular behaviour and tissue architecture. Supportive of this hypothesis, the loss of E-cadherin is a common feature of many carcinomas (Behrens, 1994), including the keratinocyte malignancy, squamous cell carcinoma (SCC) in which differentiation is suppressed and the cells become invasive (Schipper *et al.,*1991). A causative link between the absence of E-cadherin and malignant behaviour is supported by the finding that re-expressing E-cadherin in invasive carcinoma cell lines *in vitro* restores a more differentiated, non-invasive epithelial phenotype (Frixen *et al.,*1991). Furthermore, the heterogeneity in involucrin expression within a squamous cell carcinoma is exactly mirrored by the expression of E-cadherin, with involucrin only present in areas where E-cadherin is expressed (Andrews *et al.,* 1997). Keratinocytes expressing the human papilloma virus (HPV)-16 proteins E6 and E7 also fail to express functional E-cadherin, are differentiation-resistant, express high levels of the EGF receptor and are invasive. Re-expressing E-cadherin in these cells returns the EGF-R levels to normal and reverses the invasive phenotype (Wilding *et al.,* 1996). However, the relationship between the cadherins and differentiation is likely to be complex, as a recent study by Zhu and Watt (1996) shows. Contrary to expectations, the expression of a dominant negative E-cadherin in human keratinocytes inhibited proliferation and promoted differentiation.

In some malignancies, E-cadherin is not lost but some other component of the adhesion (e.g. α-catenin) may no longer be present or functional. In these cases, re-expressing the missing catenin also restores a more normal phenotype (Bullions *et al.,* 1997). Thus, there are considerable data in support of the hypothesis that cadherin-mediated adherens junctions are involved in the control of the normal epithelial phenotype although a direct demonstration that this is true has yet to be acheived.

2.3 THE SRC-FAMILY KINASES AND THE KERATINOCYTE

The Src-family is a group of nine non-receptor protein tyrosine kinases (Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk). The cellular *src* (*c-src*) gene was the first proto-oncogene to be discovered (Stéhelin *et al.,*1976) and mutations in this gene gave rise to the activated oncogenic variant *v-src* which is the transforming gene of the Rous sarcoma virus. The protein encoded by this gene, v-Src, was found to have kinase activity in 1978 (Collett and Erikson, 1978; Levinson *et al.,*1978) and c-Src was subsequently demonstrated to be a tyrosine kinase (Courtneidge and Smith, 1983; Eckhart *et al.,* 1979). Some other members of the Src family were also identified as the transforming components of avian sarcoma viruses, for example v-Yes is the transforming component of the Yamaguchi 73 avian sarcoma virus (Erikson and Erikson, 1983), whereas others were identified on the basis of homology to known members of the family, for example, Fyn (Kawakami *et al.,* 1986). Some members of the family are expressed ubiquitously, e.g. c-Src, Fyn and c-Yes, whereas others such as Lck have a more restricted tissue distribution (reviewed in Cooper, 1990). The structure of c-Src is the best characterised of the family (summarised in Figure 5 *a.*) and will now be described in more detail.

2.3.1 The structure of c-Src

The N-terminus or SH4 (Src-homology 4) domain of c-Src (Figure 5 *a*) contains the membrane anchoring region of the protein where a myristyl group is attached to the penultimate amino acid, a glycine (Kaplan *et al.,*1988). In addition to myristylation, some family members, though not c-Src, are also palmitoylated on cysteine residues in the SH4 domain strengthening their association with the plasma membrane (Koegel *et al.,*1994). However, c-Src has basic amino acids in the SH4 domain which contribute to membrane association by electrostatically interacting with acidic membrane phospholipids (Sigal *et al.,*1994). Membrane association is an important determinant of Src kinase function and is required for transformation by v-Src (Kamps *et al.,*1986).

Adjacent to the SH4 domain is the unique domain which is highly divergent between the Src family members and contributes towards the functional specificity of the different family members. For example, Lck is involved in signalling from the CD4 and CD8 receptors in T-lymphocytes and its unique domain forms disulphide bonds

with the CD8 receptor, an interaction that other family members are unable to perform (Yi and Cheong, 1996).

Following the unique domain, is the SH3 domain, which has a range of functions involving the localisation, regulation and substrate recognition of the kinase. This domain binds to proline-rich ligands that have a left-handed helical conformation (Yu *et al.,* 1994) and its binding affinity for different ligands of this type varies between Src-family members (Rickles *et al.,* 1995). c-Src also has a tyrosine phosphorylation site in the SH3 domain which decreases the affinity of the SH3 domain for ligands when it is phosphorylated, for example by the PDGF receptor (Broome and Hunter, 1995), providing an additional mechanism for regulating SH3 domain interactions.

The SH2 domain most readily binds to sequences containing phosphotyrosine in the preferred sequence YEEI (Songyang *et al.,* 1994) although not all proteins that bind to the SH2 domain contain such a sequence, for example the PDGF receptor (Kypta *et al.,* 1990; Mori *et al.,* 1993). The functions of the SH2 domain include substrate recognition and also regulation of kinase activity. Like the SH3 domain, the affinity of the SH2 domain for its ligands may be regulated by post-translational modifications, such as tyrosine phosphorylation. At present, this has only been demonstrated in Lck which is phosphorylated at tyrosine 192 (homologous to Y213 in c-Src), possibly by the cytoplasmic tyrosine kinase Syk (Couture *et al.,* 1994).

The kinase (or SH1) domain is highly conserved within the Src family and also plays a role in substrate selection. The preferred substrate sequence of the c-Src kinase domain is EEEIY$^{G}/_{E}$EFD which has some similarities to the preferred binding sequence of the SH2 domain, prompting the suggestion that the SH2 domain may bind to a target sequence before it is phosphorylated (Zhou *et al.,* 1995a). There is a tyrosine residue within the kinase domain (Y416 in avian c-Src) which is the major autophosphorylation site (Smart *et al.,* 1981; Cooper and MacAuley, 1988), the phosphorylation of which is required for the full activation of Src (Ferracini and Brugge, 1990). Dephosphorylated Y416 may somehow block substrate access to the kinase domain or alternatively, the phosphorylated form may serve to stabilise the catalytically active form of Src. In addition, Y416 phopshorylation may create a new binding site for the SH2 domains of other proteins, including the regulatory kinase Csk (section 2.3.2).

Figure 5

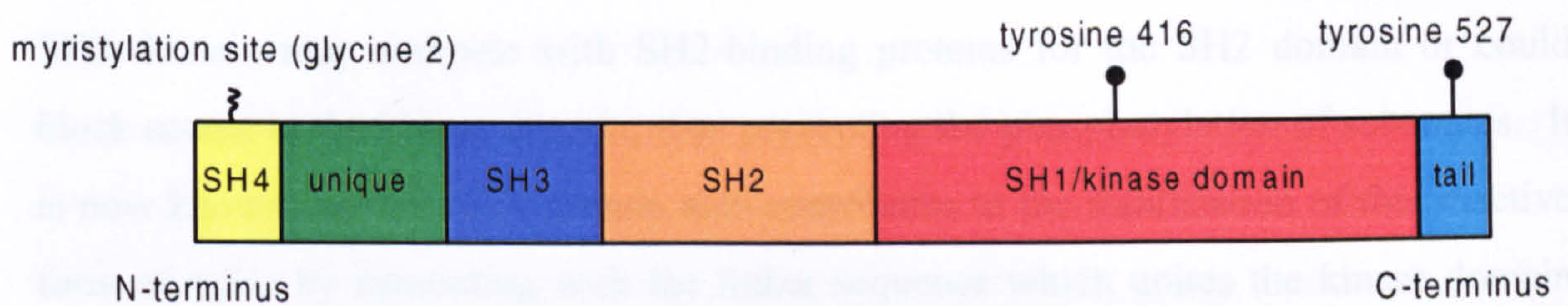
Figure 5
The structure of c-Src

a. Schematic representation of c-Src, showing the SH4 domain with membrane-anchoring myristylation site, unique domain, proline-rich sequence-binding SH3 domain, phosphotyrosine-binding SH2 domain, kinase domain with the tyrosine 416 autophosphorylation site and the carboxy-terminal tail with regulatory tyrosine 527.

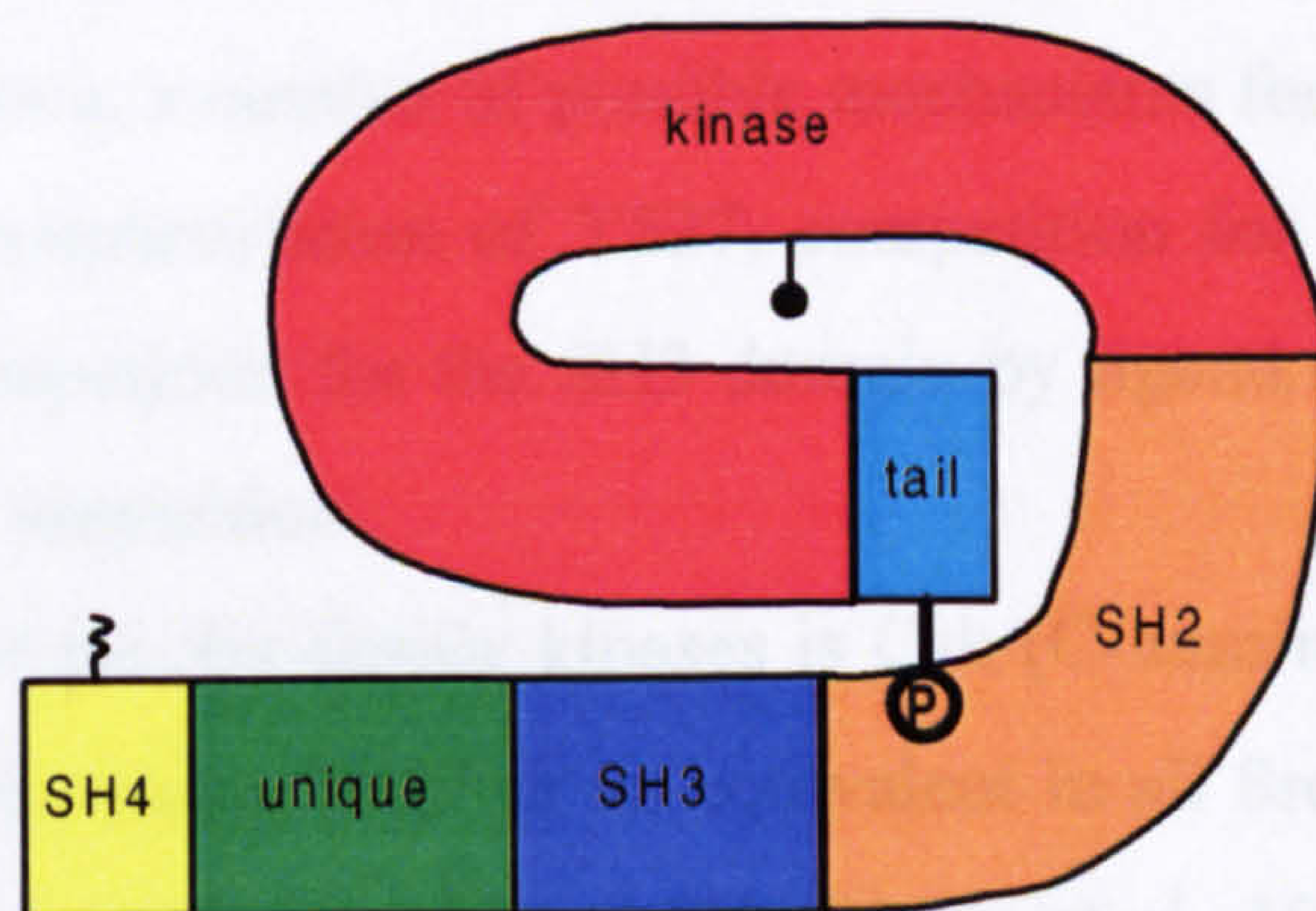
b. Schematic representation of c-Src inactivated by phosphorylation of tyrosine-527 which now interacts with the SH2 domain.

The structure of c-Src

a. The domains of c-Src



b. The conformation of inactive c-Src



The carboxy-terminal tail of the Src-family kinases is 15 to 17 residues long and is involved in the negative regulation of kinase activity. It is absent from the transforming v-Src protein. The regulatory tail contains a conserved tyrosine residue (Y527 in avian c-Src) that strongly suppresses kinase activity when it is phosphorylated (Courtneidge,1985), irrespective of the phosphorylation status of Y416 (Stover *et al.*,1994). The inhibition of Src kinase activity by phosphorylated Y527 involves the intramolecular binding of this C-terminal phosphotyrosine to the SH2 domain (Matsuda *et al.*,1990) as depicted schematically in Figure 5 *b*. This interaction causes a change in the conformation of c-Src, originally inferred from the altered pattern of proteolytic sensitivity seen in inactive c-Src (MacAuley and Cooper, 1989). Y527 binding to the SH2 domain may compete with SH2-binding proteins for the SH2 domain or could block access to the kinase domain, thus preventing the phosphorylation of substrates. It is now known that the SH3 domain also contributes to the stabilisation of this inactive form of c-Src by interacting with the linker sequence which unites the kinase domain with the SH2 domain (Xu *et al.*, 1997b). The functional significance of this interaction in Hck was recently demonstrated by its disruption with a high affinity ligand which resulted in a dramatic increase in kinase activity (Moarefi *et al.*, 1997).

2.3.2 Src-family regulation

c-Src can be activated by a range of different stimuli, including activation of the EGF and PDGF receptors (Ralston and Bishop, 1985) and elevated intracellular Ca^{++} (Rusanescu *et al.*,1995; Zhao *et al.*,1992). Considering the regulatory intramolecular interactions outlined above, a number of possible mechanisms for activating c-Src exist. These include the dephosphorylation of Y527, competition for the SH2 domain by a high affinity ligand, competition for the SH3 domain by ligand or allosteric disruption of the carboxy-tail/SH2 interaction.

A key regulator of the Src-family kinases is Csk (C-terminal Src kinase, Nada *et al.*, 1991) which phosphorylates Y527 or its equivalent in all Src-family kinases and is expressed in most tissues (Bergman *et al.*, 1992; Okada *et al.*, 1991). Csk has SH2 and SH3 domains but unlike Src, it is not membrane-localised due to the absence of a myristylation site (Nada *et al.*,1991). Although the target for Csk is Y527, mutation analyses have shown that the phosphorylation of Y527 by Csk is suppressed where the SH2 or SH3 domains are absent, suggesting that Csk interacts with other parts of Src in

addition to the C-terminal tail (Superti-Furga *et al.,*1993). Csk has high affinity for binding Y416 which may function to direct Csk to activated Src, although binding to Y416 does not seem to be essential for Csk function (Sabe *et al.,*1994).

The normal mechanism of the regulation of Csk function may not involve altering its activity but, instead, may depend on varying its subcellular localisation, thus determining its access to Src-family kinases. These changes in subcellular localisation could be brought about by interactions between Csk and tyrosine-phosphorylated proteins that may target Csk to areas where Src is active (Howell and Cooper, 1994). This is supported by the finding that membrane-targeted Csk suppresses c-Src activity more effectively than normal Csk, demonstrating that Csk is most effective when localised to the same subcellular compartment as the active kinase (Chow *et al.,* 1993). Two phosphatases (Syp and SHP-1) which can counteract the effects of Csk by dephosphorylating Y527, thus activating c-Src, have now been identified. Both phosphatases are phosphorylated by, and bind to c-Src (Peng and Cartwright, 1995; Somani *et al.,*1997).

Mice with the Csk -/- genotype die during embryogenesis although development is normal up to gastrulation, suggesting that maternal Csk may persist up until that time or that another kinase can partially substitute for Csk (Imamoto and Soriano, 1993; Nada *et al.,* 1993). Furthermore, tyrosine 527 is partially phosphorylated in Csk -/- cells (Imamoto and Soriano, 1993) and although there is some evidence that Src can autophosphorylate at Y527 (Osusky *et al.,* 1995), when kinase defective Src is expressed in these cells it is also phosphorylated on Y527. A Csk relative has now been identified, namely Chk (Csk homologous kinase), previously referred to as Lsk, Hyl and Matk (Grgurevich *et al.,* 1997), which can partially substitute for Csk in Csk -/- fibroblasts (Davidson *et al.,* 1997). Chk is probably the only other kinase that can phosphorylate Y527.

2.3.3 Src-family substrates

A plethora of Src-family substrates have been identified, including Shc (McGlade *et al.,*1992), p190 (Ellis *et al.,* 1990), the regulatory p85 subunit of PI3-kinase (Haefner *et al.,* 1995; Liu *et al.,* 1993) and numerous components of focal adhesions including pp125^{FAK} (Calalb *et al.,* 1995; Schaller *et al.,* 1994), paxillin (Weng *et al.,* 1993), tensin (Davis *et al.,* 1991), talin (Pasquale *et al.,* 1986) and vinculin (Sefton *et al.,* 1981). In

addition some substrates are components of cell-cell adhesions, for example vinculin and the catenin $p120^{ctn}$ (section 2.2.2 and Reynolds *et al.*, 1989). The interactions between Src kinases and $p120^{ctn}$ are not understood but a direct association involving the Src SH2 or SH3 domains is thought to be unlikely. More probable, is a common membrane localisation as a result of myristylation, a modification that is required for $p120^{ctn}$ phosphorylation by v-Src in fibroblasts (Linder and Burr, 1988). The phosphorylation of $p120^{ctn}$ by Src does not result in the displacement of $p120^{ctn}$ or the cadherins from the adhesions (Reynolds *et al.*, 1994) and is perhaps more likely to interfere with the interactions between the adhesion complex and the actin cytoskeleton.

2.3.4 Src kinases in the epidermis

Three members of the Src family are expressed in the epidermis- c-Src, c-Yes and Fyn. c-Src is abundant within the perinuclear cytoplasm of spinous and granular layer keratinocytes (Zhao *et al.*, 1992) whereas c-Yes is abundant in the keratinocytes of the basal layer and is also present, although to a lesser degree, in the spinous layers (Krueger *et al.*, 1991). Neither c-Yes nor c-Src are detectable in the cornified layer, probably a consequence of the intracellular degradative processes that take place during the transition between the granular and cornified layers. The localisation of Fyn within the epidermis has yet to be determined.

There are now reports of all three kinases playing roles in the keratinocyte differentiation process, obtained using *in vitro* culture systems (section 3.1) and one using mice nullizygous for the *fyn* gene. In human keratinocytes maintained *in vitro*, c-Src kinase activity is rapidly and transiently increased in keratinocytes treated with the ionophore A23187 in the presence of 1.0mM extracellular Ca^{++} (Zhao *et al.*,1992). This is a non-physiological treatment which increases the intracellular Ca^{++} concentration, thus inducing the keratinocyte transglutaminase to assemble cornified envelopes, partially recreating the granular to cornified layer transition. Although envelope assembly *in vivo* is also accompanied by Ca^{++} influx, it is unclear how closely ionophore treatment resembles the physiological situation, especially in view of the observation that ionophore treatment causes a similar increase in c-Src kinase activity in fibroblasts (Zhao *et al.*,1993). Under identical experimental conditions, the activity of c-Yes gradually decreases, possibly as a result of the Ca^{++}-dependent binding of an inhibitory protein upon ionophore treatment (Zhao *et al.*,1993).

The evidence supporting a role for Fyn in the control of keratinocyte differentiation is perhaps more compelling, as similar results were obtained both *in vitro* and *in vivo* (Calautti *et al.,* 1995) using *fyn* -/- mice and keratinocytes. In cultured murine *fyn* -/- keratinocytes, the differentiation response to high Ca^{++} (section 3.2.6) was abnormal and the induction of some differentiation-specific proteins including filaggrin and keratin 1 (K1) was suppressed, as was inducible transglutaminase activity. In addition, the delayed/reduced induction of filaggrin and K1 was also seen in the epidermis of *fyn* -/- mice. Interestingly, no such defects were seen in c-Src -/- mice or keratinocytes. Thus, there is evidence that members of the Src family may be involved in the regulation of keratinocyte differentiation although their precise roles in this process remain to be explored.

2.4 Protein Kinase C and the keratinocyte

2.4.1 The protein kinase C family - regulation and substrates

The protein kinase C family of phospholipid-dependent serine/threonine protein kinases comprises at least 12 different mainly soluble isozymes, all requiring phosphatidylserine for activity but each having distinct functional chararacteristics. The conventional α, β and γ protein kinase C (PKC) isozymes require both Ca^{++} and 1,2-diacylglycerol as cofactors for full activity although phorbol esters (section 3.2.1) can substitute for diacylglycerol (Nishizuka, 1984; Castagna *et al.,* 1982). Conversely, the novel PKC isozymes (δ, ε, η and σ) do not require the presence of Ca^{++} for full activation (e.g. Kiley *et al.,* 1990). Other PKC isozymes such as ζ and λ are considered atypical forms of the enzyme as they do not require Ca^{++} or diacylglycerol (Akimoto *et al.,* 1994). All of the PKC isozymes have an N-terminal regulatory domain containing phosphatidylserine and phorbol ester binding sites.

Each of the PKC isozymes phosphorylates different substrates in the cell. However, when purified and tested against a range of substrates *in vitro*, a lack of substrate specificity is evident (Kazanietz *et al.,* 1993). This suggests that some mechanism other than the intrinsic substrate specificity of the enzyme determines which substrates are phosphorylated. The basis of this selectivity is thought to involve the

specific subcellular localisation of the different isozymes thus determining substrate availability. In addition, the distinct subcellular localisations allow these predominantly soluble enzymes to phosphorylate cytoskeletal and membrane-bound proteins. A range of proteins to which PKC isozymes bind and could participate in this targeting process (and may also be substrates) have been identified, including the actin-binding proteins vinculin and talin (Hyatt *et al.,* 1994). It was not demonstrated that these proteins were substrates of PKC in this study, but it was demonstrated that PKCα binding could take place in the absence of Ca^{++}, which is normally required for the catalytic activity of this enzyme. These data suggest that the binding activity and catalytic activity of PKC are separate functions.

2.4.2 Protein kinase C isozymes and keratinocyte behaviour

The tumour-promoting effects of phorbol esters, when applied to murine epidermis strongly implicate members of the PKC family in the control of keratinocyte transformation. Although this is almost certainly true, non-protein kinase C receptors for phorbol esters also exist in some cell types (Ahmed *et al.,* 1993). Keratinocytes have yet to be investigated for these novel receptors.

A number of protein C isozymes are present in the epidermis, including the Ca^{++}-dependent PKCα and PKCβ isozymes and the Ca^{++}-independent δ, ε, η and ζ isozymes (Fisher *et al.,* 1993). Within the adult epidermis, PKCα is expressed mainly in the lower epidermis, PKCβ is predominantly localised to the upper cell layers and the Ca^{++}-independent isozyme PKCη, is highly expressed in the suprabasal layers (Osada *et al.,* 1993). Interestingly, this study also found that PKCβ is virtually absent from psoriatic epidermis. Overexpressing PKCη in the suprabasal layers of murine epidermis stimulates squamous differentiation whereas the similar expression of a dominant negative mutant of PKCη arrests differentiation in the granular layer (Kuroki *et al.,* 1997). Together these data provide convincing evidence for PKC isozymes having an important role in keratinocyte differentiation.

Altering keratinocyte PKC activity *in vitro* has been shown to modulate the stability of both adherens junctions (section 2.2.2; Lewis *et al.,* 1994a; Citi, 1992) and desmosomes (Kitajima *et al.,* 1992) and there is also evidence that PKC may regulate focal adhesion turnover in a hamster melanoma cell line (Lewis *et al.,* 1996). In

addition, the 1,25-dihydroxyvitamin D3-induced formation of cadherin-mediated keratinocyte intercellular adhesions *in vitro* is supressed by protein kinase C inhibitors, again suggesting that this kinase may play a role in the assembly of this type of adhesion (Gniadecki *et al.*, 1997).

CHAPTER 3

THE *IN VITRO* STUDY OF KERATINOCYTE DIFFERENTIATION

3.1 KERATINOCYTE CULTURE SYSTEMS

The ability to propagate normal and malignant epidermal keratinocytes *in vitro* has greatly increased our understanding of the differentiation process. Many features of differentiation can be artifically induced in a population of keratinocytes under conditions where detailed biochemical analyses are possible, thus allowing a fuller understanding of the processes involved. A number of different keratinocyte culture systems exist, each with distinct advantages and disadvantages as outlined below.

3.1.1 Growth in the presence of serum

The first systems that could successfully maintain isolated keratinocytes (as opposed to whole skin explants) for significant periods of time *in vitro* were reported by Rheinwald and Green in 1975. They used a feeder layer of 3T3 fibroblasts prevented from proliferating by lethal irradiation, to produce a cocktail of growth factors and extracellular matrix proteins (Alitalo *et al.*, 1982) allowing the keratinocytes to be propagated in serum-containing medium. In this culutre system, the keratinocytes do not grow as a monolayer but rather as a series of stratified (multilayered) colonies which in some respects resemble the epidermis. For example, proliferation only occurs in the 'basal' layer adjacent to the tissue culture substrate and the upper layers of the colonies express proteins that are restricted to the differentiating keratinocytes of the suprabasal layers in the skin (Watt, 1988).

3.1.2 Growth on a floating dermal equivalent

A very different type of culture system which more fully recreates the structure of the epidermis *in vitro* is the 'organotypic' raft culture where keratinocytes are seeded onto floating dermal equivalents. The cells grow at the air-liquid interface of the culture and produce a stratified structure very similar to normal epidermis (Régnier, 1981). A dermal equivalent consists of a section of de-epidermalised dermal tissue or extracellular matrix such as collagen (Kopan *et al.*,1987; Karasek and Charlton, 1971).

This culture system recreates the physiological differentiation process more fully than any other keratinocyte growth system, but a major difficulty is that cells at different stages of differentiation cannot be isolated for biochemical analyses.

3.1.3 Growth in the absence of serum

A third type of culture system used for propagating human keratinocytes does not make use of feeder cells and does not contain serum, thus removing some of the complicating factors that can hamper the interpretation of studies performed using the culture system described by Rheinwald and Green. These serum-free culture systems are based on the MCDB 153 medium to which bovine pituitary extract and some defined growth factors (hydrocortisone, insulin, EGF) are added for optimal growth (Tsao *et al.*, 1982). A further advantage of these culture systems is that they can easily be prepared to contain different amounts of Ca^{++} which greatly facilitates the study of the consequences of altering the extracellular Ca^{++} concentration on the control of keratinocyte behaviour (section 3.2.6).

3.2 MODULATORS OF KERATINOCYTE DIFFERENTIATION

A variety of agents and culture conditions which can modulate the differentiation status of keratinocytes *in vitro* have been defined using the different culture systems outlined above. These include phorbol esters, retinoids, dihydroxyvitamin D3, growth in suspension, cell shape and extracellular Ca^{++}.

3.2.1 Phorbol esters

Phorbol esters are a group of compounds that are potent tumour promoters when applied to murine skin (reviewed in Scribner and Suss, 1978); however, they can also induce features of the differentiation process *in vitro*. Phorbol, 12-myristate, 13-acetate (PMA) is a phorbol ester that induces some aspects of human keratinocyte differentiation *in vitro* including altered cell morphology, altered adhesion, cytoplasmic and nuclear destruction and the assembly of cornified envelopes (Parkinson and Emmerson, 1982). Furthermore, there is also evidence from studies on murine keratinocytes that the PMA can act to induce changes indicative of the spinous to

granular layer transition, including the expression of loricrin and filaggrin whilst repressing spinous cell differentiation proteins such as keratins K1 and K10 (Dlugosz and Yuspa, 1993; Dlugosz and Yuspa, 1994). This process is likely to involve the activation of protein kinase C (PKC) by PMA since these authors showed that inactivating PKC using GF109203X, blocked the effects of PMA on differentiation. GF109203X is a selective inhibitor of PKCα, β_I, β_{II}, γ, δ and ε which acts by competetive inhibition of ATP binding (Toullec *et al.,* 1991).

3.2.2 Retinoids

Retinoids such as all *trans*-retinoic acid can inhibit differentiation in keratinocytes (Monzon *et al.,*1996; Gibbs *et al.,*1996) although they have the opposite effect on some other cell types (Rodrigues *et al.,*1985; Jetten *et al.,*1979). Retinoids are present in normal human epidermis where they are likely to play a role in the regulation of epidermal proliferation and differentiation (Vahlquist, 1982). There are also a number of receptors for retinoic acid in the epidermis and the specific receptors present in a cell change during the differentiation process, suggesting that keratinocytes can respond differently to retinoic acid depending on their state of differentiation (Reichrath *et al.,*1995; Eller *et al.,*1994; Elder *et al.,* 1992; Vollberg *et al.,* 1992). Retinoic acid can induce changes in transcription when complexed with its receptor and has been shown to suppress the promoters of keratins K3, K5, K10, K14 and K16 *via* its nuclear receptor (Blumenberg *et al.,* 1992).

3.2.3 Dihydroxyvitamin D3

Vitamin D3 is synthesised in the skin in response to ultraviolet light (Takada *et al.,*1979) and is then metabolised to the active form, 1,25-dihydroxyvitamin D3 or calcitrol, which affects keratinocyte growth and differentiation *in vitro.* Low, physiological concentrations of less than 10^{-9} M enhance proliferation whereas concentrations higher than 10^{-8} M cause cell-cycle arrest and enhance differentiation in response to stimuli such as Ca^{++} (section 3.2.6) (Itin *et al.,* 1994). In addition, it has been shown that 1,25-dihydroxyvitamin D3 inhibits the proliferation of undifferentiated keratinocytes whilst stimulating the proliferation of differentiated cells *in vitro* (Gniadecki, 1996).

In keratinocytes, vitamin D3 receptor expression changes according to the differentiation status of the cells *in vitro*, with more receptor expressed in the less differentiated cells (Pillai *et al.*,1988a). The mechanism of action of 1,25-dihydroxyvitamin D3 may be similar to that of retinoic acid since the nuclear receptors for 1,25-dihydroxyvitamin D3 and retinoic acid recognise the same DNA regulatory elements (Schule *et al.*, 1990), although it is not thought to affect keratin expression directly (Tomic *et al.*, 1992).

3.2.4 Growth in suspension

The induction of keratinocyte differentiation *in vitro* by depriving the cells of cell-substratum contact was one of the first model systems used to study differentiation (Green, 1977). This technique involves suspending the cells in methyl-cellulose, preventing the formation of both cell-cell and cell-matrix contacts and induces many aspects of differentiation including loss of integrin function, cell-cycle withdrawal and involucrin expression (Nicholson and Watt, 1991; Adams and Watt, 1990; Watt *et al.*, 1988). This induction of differentiation is a consequence of the lack of integrin-mediated attachment since the presence of soluble fibronectin in the methyl-cellulose inhibits differentiation (Adams and Watt, 1989).

The relevance of this model system to *in vivo* differentiation is unclear. Although keratinocytes would not normally be totally isolated from their surroundings in this way, the severing of integrin-mediated contacts with the basement membrane is an early feature of the differentiation process (section 2.1). However, it also possible that suspension in methyl-cellulose induces 'anoikis' (cell death in an inappropriate environment, section 2.1), of which partial differentiation could be a consequence. In addition, the observation that continued occupation of the fibronectin receptor blocks the induction of differentiation, possibly by providing a survival signal, may not be applicable to keratinocytes *in vivo* which only express the integrin $\alpha_5\beta_1$ fibronectin receptor in areas of wound healing (Cavani *et al.*,1993; Hertle *et al.*,1992). However Watt *et al.*, (1993) showed that a combination of collagen IV and laminin in the presence of a suboptimal fibronectin concentration was also sufficient to inhibit suspension-induced differentiation *in vitro*.

3.2.5 Cell shape

Keratinocytes in suspension culture assume a different morphology from adherent cells and this could contribute to the induction of differentiation. Also, some of the treatments which induce differentiation *in vitro*, e.g. Ca^{++} (section 1.7.2) cause changes in cell shape. Thus, cell shape may play a role in modulating keratinocyte differentiation. A study which sought to answer this question used small adhesive islands surrounded by a non-adhesive surface to regulate the shape of adherent keratinocytes (Watt *et al.,* 1988). This approach showed that decreasing the area of cell-substratum contact increased the probability of cell-cycle arrest and differentiation. However, it is difficult to separate the effects of altered cell shape from cell-matrix contact area with such a method and it is possible that the effects observed were due to the reduction of integrin-matrix contacts below a critical threshold, analagous to the situation in methyl-cellulose suspension cultures. The effects of cell shape on commitment to differentiation in the basal layer are difficult to determine since most cells in the basal layer are similarly columnar in shape.

3.2.6 High extracellular calcium

It is well established that extracellular calcium ions (Ca^{++}) can modulate keratinocyte differentiation. This is especially true for the serum-free culture system in which the Ca^{++} concentration is more easily controlled than in the Rheinwald and Green serum-containing culture system (Boyce and Ham, 1983). Low extracellular Ca^{++} concentrations (less than 0.1mM) inhibit differentiation whereas higher Ca^{++} concentrations (greater than 0.3mM) stimulate differentiation (Pillai *et al.,* 1988b).

A. Induction of differentiation by calcium

High extracellular Ca^{++} induces the production of a number of changes characteristic of differentiation, including the expression of, filaggrin (Dale *et al.,*1983), involucrin and keratinocyte transglutaminase (Pillai *et al.,*1988b). In fact, exposing keratinocytes to high extracellular Ca^{++} for a period longer than 24 hours is sufficient not only to induce the expression of selected differentiation-specific proteins but also to increase the number of cells that are competent to assemble cornified envelopes (Pillai *et al.,*1988a). This observation suggests that high extracellular Ca^{++} must induce a sufficient range of envelope components to enable envelope assembly to take place.

B. Modulation of cell adhesion by Ca^{++}

In low extracellular Ca^{++} cell-cell adhesion is suppressed and the keratinocytes have a scattered distribution on the substrate, interacting with one another only weakly. Conversely, in high extracellular Ca^{++}, the cells grow in tightly packed, stratified 'islands', similar to those seen in Rheinwald and Green cultures (which have an extracellular Ca^{++} concentration of 1.8mM), surrounded by areas of substratum that are often devoid of cells (Pillai *et al.,* 1988b). These groups of keratinocytes partially recreate the structural organisation of the epidermis in that the most differentiated cells are furthest from the substrate in the upper layers of the aggregate. These changes in distribution correlate with changes in the subcellular distribution of the cadherins (Lewis *et al.,*1994b), integrins (Symington *et al.,* 1993) and desmosomal components (Watt, 1984) (sections 2.2.2, 2.1 and 2.2.1). It has been shown that it is the cadherins which mediate the initial Ca^{++}-induced adhesion and stratification and that they are required for the subsequent assembly of desmosomes (Amagai *et al.,*1995; Lewis *et al.,*1994b; Wheelock and Jensen, 1992). In high Ca^{++}, the keratin cytoskeleton rearranges to interact with these newly formed desmosomes (e.g., Amagai *et al.,*1995). A direct consequence of the formation of cadherin-mediated cell-cell adhesions is the perturbation of the cell-surface integrins on involucrin-positive cells which intially lose function and are then internalised (Hodivala and Watt, 1994), contributing to the distinctive spatial organisation that is seen in high extracellular Ca^{++}.

C. Effects of Ca^{++} on proliferation

There are conflicting reports on the effects of high extracellular Ca^{++} on keratinocyte cell-cycle progression (section 3.3) *in vitro.* Some authors have reported that there is no significant effect on proliferation (Poumay and Pittelkow, 1995), other reports showed that proliferation is inhibited by high extracellular Ca^{++} (Pillai *et al.,*1988a; Hawley-Nelson *et al.,* 1980) whilst others showed that proliferation was promoted by high extracellular concentrations (Al-Ani *et al.,* 1988; Boyce and Ham, 1983).

D. Mechanisms of Ca^{++} action

To date, a clear consensus on the way in which high extracellular Ca^{++} induces aspects of differentiation has not been reached. A potential mechanism for the action of

high extracellular Ca^{++} is the modulation of intracellular Ca^{++} levels. Shifting to high extracellular Ca^{++} causes the intracellular Ca^{++} concentration of murine keratinocytes to increase (Hennings *et al.,* 1989); furthermore, supressing this increase with intracellular Ca^{++}-chelating agents abrogates the effect of high extracellular Ca^{++} on differentiation (Li *et al.,* 1995). These data strongly suggest that the intracellular Ca^{++} concentration has a profound influence on the behaviour of murine keratinocytes; however, the situation in human keratinocytes is less clear. The intracellular Ca^{++} concentration in human keratinocytes does increase by a small amount in response to elevated extracellular Ca^{++} although the functional significance, if any, of this change has yet to be elucidated (Watt *et al.,* 1991). Keratinocytes may sense the extracellular Ca^{++} concentration through the extracellular divalent cation receptor which was identified in murine keratinocytes (Filvaroff *et al.,* 1994) which is believed to trigger the rise in intracellular Ca^{++} when the extracellular Ca^{++} concentration is raised (Bikle *et al.,*1996). Additionally, the changes in cell-cell and cell-substratum adhesion that are induced by high extracellular Ca^{++} could also play a role in the induction of differentiation. The formation of new adhesions, or the breaking of existing adhesions could trigger signalling events that modulate differentiation. The importance of Ca^{++} with respect to differentiation in the skin is unknown. A Ca^{++} concentration gradient exists in the skin, with a relatively low concentration in the basal layer, increasing to a maximal concentration in the granular layer (Menon *et al.,* 1985) but the role this plays has yet to be determined.

MATERIALS AND METHODS

CHAPTER 4

MATERIALS

Suppliers are listed alphabetically in each section and UK importers details are given in parentheses where appropriate.

4.1 Human tissue

Supplier: ***The Royal Hospital for Sick Children, Yorkhill, Glasgow.***

Freshly excised human foreskin tissue.

4.2 Cell culture reagents

Supplier: ***Advanced Protein Products, Brierley Hill, UK.***

Foetal calf serum

Supplier: ***Beatson Institute Central Services***

Sterile dH_2O

Sterile PBS (Dulbecco 'A')

Sterile PBS/1mM EDTA

Penicillin solution (10,000 units/ml)

Streptomycin solution (10mg/ml)

Supplier: ***Clonetics Corporation (TCS Biologicals, Botolph Claydon, UK.).***

Serum-free, calcium-free keratinocyte basal medium (KBM)

Keratinocyte growth medium (KGM) SingleQuot kit:

- 2ml bovine pituitary extract, 7.5mg/ml
- 0.5ml human epidermal growth factor, 0.1μg/ml
- 0.5ml insulin, 5mg/ml
- 0.5ml hydrocortisone, 0.5mg/ml

0.5ml GA-1000 (gentamicin/amphotericin, 50mg/ml and 50μg/ml)

2ml calcium chloride solution, 300mM

Supplier: ***Gibco Europe Life Technologies Ltd., Paisley, UK.***

10× Dulbeccos MEM concentrate

7.5% (w/v) sodium bicarbonate

100mM sodium pyruvate

200mM L-glutamine

2.5% trypsin solution

Supplier: ***Sigma Chemical Co., Poole, UK.***

Type II-T trypsin inhibitor (from turkey egg white)

histidine

isoleucine (allo-free)

methionine

phenylalanine

tryptophan

tyrosine

Supplier: ***Worthington Biochemical Corp. (Lorne Laboratories Ltd., Reading, UK.).***

2× trypsin (lyophilised)

4.3 Cell culture plasticware

Supplier: ***A/S Nunc (TCS Limited, Botolph Claydon, UK.).***

Nunc Delta tissue culture flasks

Nunc chamber slides

Nunc cryotubes

Supplier: ***Beckton Dickinson Labware, Plymouth, UK.***

Falcon tissue culture dishes

Supplier: ***Costar Corp., Cambridge, MA, USA.***

96-well tissue culture plates

Cell scrapers

4.4 INHIBITORS, PEPTIDES AND IONOPHORES

Supplier: ***Affiniti Research Products, Mamhead Castle, UK.***

Synthetic decapeptides, >95% purity:

LRAHAVDVNG-amide

VIPPINLPEN-amide

Supplier: ***Calbiochem, Nottingham, UK.***

Ionomycin (free acid) from *Streptomyces conglobatus*

Supplier: ***Alan J. Kraker at Parke-Davis Pharmaceuticals, Ann Arbor, MI, USA.***

PD162531 Src-family kinase inhibitor

Supplier: ***Sigma Chemical Co., Poole, UK.***

Cytochalasin D

Nocodazole

4.5 CELL VIABILITY ASSAYS

Supplier: ***Promega, Southmpton, UK.***

CellTiter 96 non-radioactive cell proliferation assay

4.6 ANTISERA AND ASSOCIATED MATERIALS

Supplier: ***Amersham International, Little Chalfont, UK.***

Sheep anti-mouse/horseradish peroxidase conjugate

Donkey anti-rabbit/horseradish peroxidase conjugate

Supplier: ***Amicon Inc., Stonehouse, UK.***

Centricon-10 concentrators

Supplier: ***Beckton Dickinson Labware, Plymouth, UK.***

β_1-integrin antiserum, rat monoclonal (mAb 13)

Supplier: ***Biomedical Technologies Inc. (Biogenesis Ltd, Poole, UK.).***

Human filaggrin antiserum, mouse monoclonal IgG1 (BT-576)

Human transglutaminase antiserum, mouse monoclonal B. C1 (BT-621)

Supplier: ***Calbiochem, Nottingham, UK.***

c-Src antiserum, mouse monoclonal IgG1 (mAb 327)

Supplier: ***Jackson ImmunoResearch, Luton, UK.***

FITC labelled sheep anti-mouse IgG

Supplier: ***Santa Cruz Biotechnology Inc. (Autogen Bioclear UK Ltd., Calne, UK.).***

Fyn antiserum, rabbit polyclonal IgG (Fyn(3))

Supplier: ***Sigma Chemical Co., Poole, UK.***

Actin antiserum, mouse monoclonal (AC-40)

E-cadherin antiserum, rat monoclonal DECMA-1

β-catenin antiserum, rabbit polyclonal

Involucrin antiserum, mouse monoclonal IgG1 (SY5)

α-tubulin antiserum, mouse monoclonal IgG1 (DM 1A)

Rabbit anti-rat/horseradish peroxidase conjugate

FITC labelled goat anti-rat IgG (affinity isolated)

FITC labelled goat anti-rabbit (IgG fraction)

FITC labelled goat anti-rat IgG

Normal mouse IgG

Rabbit anti-mouse IgG

Protein A-sepharose beads

Supplier: ***Transduction Laboratories (Affiniti Research Products Ltd., Mamhead, UK.).***

E-cadherin antiserum, mouse monoclonal IgG2a (Clone #36)

P-cadherin antiserum, mouse monoclonal IgG1 (Clone #56)

$p120^{ctn}$ antiserum, mouse monoclonal IgG1 (Clone #98)

Phosphotyrosine antiserum, mouse monoclonal IgG2b (PY20)

c-Yes antiserum, mouse monoclonal IgG1 (Clone #1)

Supplier: ***Vector Laboratories, Peterborough, UK.***

Fluorescein labelled PNA (peanut agglutinin) from *Arachis hypogaea*

4.7 FLOW CYTOMETRY REAGENTS

Supplier: ***Boehringer Mannheim, Lewes, UK.***

RNAse A

Supplier: ***Cadisch and Sons, Finchley, UK.***

Nylon mesh (70µm pore size)

Supplier: ***Sigma Chemical Co., Poole, UK.***

Propidium iodide

4.8 Protein electrophoresis and immunoblotting

Supplier: ***Amersham, Little Chalfont, UK.***

High molecular weight rainbow markers (14.4kDa to 220kDa)

Supplier: ***BDH Laboratory Supplies, Poole, UK.***

Ammonium peroxodisulphate (APS)

Supplier: ***Beatson Institute Workshop.***

Semi-dry blotting apparatus

Supplier: ***Genetic Research Instrumentation, Dunmow, UK.***

Atto protein electrophoresis apparatus

Supplier: ***Pierce (Life Science Laboratories Ltd., UK.)***

Micro BCA (Bicinchoninic acid) protein assay kit

Supplier: ***Schleicher and Schuell, London, UK.***

Protran BA85 nitrocellulose membrane

Supplier: ***Severn Biotech Ltd., Kidderminster, UK.***

Design-a-Gel 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution

Supplier: ***Sigma Chemical Co., Poole, UK.***

TEMED (N, N, N′, N′-Tetramethylethylenediamine)

0.1% (w/v) Ponceau S solution in 5% acetic acid

Bovine serum albumin (BSA), fraction V

Supplier: ***Technical Photo Systems, Cumbernauld, UK.***

Fuji RX X-ray film

Supplier: ***Unipath, Basingstoke, UK.***

'Oxoid' phosphate buffered saline tablets (Dulbecco 'A')

Supplier: ***Whatman, Maidstone, UK***

3MM filter paper

4.9 Tyrosine kinase assay reagents

Supplier: ***Amersham, Little Chalfont, UK.***

[γ-^{32}P]ATP, redivue, 3000Ci/mmol

Supplier: ***Beckman Instruments Inc., High Wycombe, UK.***

Liquid scintillation counter, model LS 60001C

Supplier: ***National Diagnostics (B.S. & S. (Scotland) Ltd., Edinburgh, UK.).***

Ecoscint aqueous scintillant

Supplier: ***Packard Instruments B.V., Groningen, The Netherlands.***

Scintillation vials

Supplier: ***Sigma Chemical Co., Poole, UK.***

ATP (Adenosine 5'-trisphosphate)

poly(Glu,Tyr) (4:1) synthetic peptide

Supplier: ***Whatman, Maidstone, UK.***

p81 chromatography paper

4.10 Immunofluorescence and immunoperoxidase staining materials

Supplier: ***Sigma Chemical Co., Poole, UK.***

3,3′-diaminobenzidine tetrahydrochloride (10mg) tablets (DAB)

Supplier: ***Vector Laboratories Ltd., Peterborough, UK.***

Vectashield mounting medium for immunofluorescence

Vectastain ABC staining kit (mouse peroxidase)

4.11 Stock solutions and buffers

4.11.1 Cell culture solutions

Keratinocyte growth medium (KGM)

Clonetics serum-free, calcium-free keratinocyte basal medium, supplemented as follows:

30μg/ml bovine pituitary extract

10ng/ml human epidermal growth factor (recombinant)

0.5μg/ml hydrocortisone

5μg/ml insulin

50ng/ml amphotericin-B

50μg/ml gentamicin

0.03mM (LKGM) or 1.00mM (HKGM) $CaCl_2$

Amino-acid enriched keratinocyte growth medium

As for normal KGM with the addition of the following supplements:

50.300mg/l	histidine
5.904mg/l	isoleucine
13.428mg/l	methionine
14.868mg/l	phenylalanine
9.180mg/l	tryptophan
8.104mg/l	tyrosine

The addition of these supplements increased the concentration of the amino acids by a factor of 4.

10C cell culture medium

1× DMEM supplemented as follows:

10% (v/v) foetal calf serum

0.225% (w/v) sodium bicarbonate

1mM sodium pyruvate

2mM L-glutamine

50 units/ml penicillin

10μg/ml streptomycin

Trypsin solution for routine passaging

0.25% Gibco trypsin in sterile PBS/1mM EDTA

Trypsin inhibitor solution

2.5mg/ml type II-T trypsin inhibitor and 1mg/ml BSA in sterile PBS

4.11.2 Protein extraction and processing

CSK lysis buffer

10mM PIPES, pH 6.8

50mM NaCl

3mM $MgCl_2$

300mM sucrose

0.5% (v/v) Triton X-100

10mM sodium pyrophosphate

2mM phenylmethylsulphonyl fluoride

100μM sodium orthovanadate

0.1% (v/v) aprotinin

RIPA buffer

50mM Tris/HCl, pH 7.6

150mM NaCl

1% Triton X-100

0.5% deoxycholate

0.1% sodium dodecyl sulphate (SDS)

2mM EGTA

10mM sodium pyrophosphate

2mM phenylmethylsulphonyl fluoride

100μM sodium orthovanadate

0.1% (v/v) aprotinin

3× sample buffer

150mM Tris/HCl, pH 6.7

6% (w/v) SDS

30% (v/v) glycerol

15% (v/v) 2-mercaptoethanol

bromophenol blue to colour

SDS-PAGE stacking gel buffer

0.5M Tris/HCl, pH 6.7

0.4% (w/v) SDS

Stacking gel:

4.0ml 30% acrylamide/0.8% bisacrylamide solution

6.0ml stacking gel buffer

14.0ml dH_2O

250μl 10% APS solution

20μl TEMED

SDS-PAGE resolving gel buffer

1.5M Tris/HCl, pH 8.9

0.4% (w/v) SDS

Resolving gel (for a 7.5% gel):

9.0ml 30% acrylamide/0.8% bisacrylamide solution

9.0ml resolving gel buffer

18.0ml dH_2O

250µl 10% APS solution

20µl TEMED

Protein electrophoresis tank buffer (10× concentrate)

1M Tris

1M glycine

2% (w/v) SDS

Semi-dry transfer buffer for immunoblotting

48mM Tris

39mM glycine

0.037 % (w/v) SDS

20% methanol

Immunoblot wash buffer

0.2% Tween 20 in PBS

Immunoblot stripping buffer

0.2M glycine

1% (w/v) SDS

pH adjusted to 2.5 with hydrochloric acid

Tyrosine kinase assay buffer

100mM PIPES, pH 6.8

20mM $MnCl_2$

10μM sodium orthovanadate

4.11.3 Immunofluorescence and immunoperoxidase cell staining

Immunofluorescence buffer (KRH)

25mM HEPES, pH 7.4

120mM NaCl

6mM KCl

1.2mM $MgCl_2$

1mM $CaCl_2$

0.05% (v/v) Tween 80

Immunoperoxidase buffer (high salt PBS)

PBS supplemented with 150mM NaCl

pH adjusted to 7.6 with NaOH

Immunoperoxidase substrate solution

16.7ml PBS

1 DAB tablet

10μl hydrogen peroxide (30% solution)

Immmunoperoxidase cover-slip mounting solution

50% (v/v) glycerol in PBS

0.025% (w/v) sodium azide

CHAPTER 5

METHODS

5.1 CELL CULTURE

5.1.1 Preparation of keratinocytes from human tissue

Freshly excised human foreskin tissue was stored at 4°C in 10C medium (4.11.1) before isolating keratinocytes as follows:

Firstly, the tissue was rinsed with PBS and was then cut into narrow strips using a scalpel. These strips of tissue were incubated in 0.125% (w/v) Worthington trypsin solution in PBS (100ml per specimen) at 4°C overnight. The following day, the epidermis was scraped off the underlying tissue using a scalpel and the resulting epidermal fragments were pipetted gently in ice-cold 10C medium to disaggregate the cells and neutralise the trypsin. The cells were then pelleted by centrifugation (1000rpm for 5 minutes) and washed twice with ice-cold low Ca^{++} KGM before plating in low Ca^{++} KGM at an initial density of 5×10^3 cells/cm^2 in 9cm culture dishes. These cultures were then maintained in a humid 37°C/5% CO_2 incubator.

5.1.2 Routine keratinocyte propagation

Adherent keratinocytes were routinely grown on tissue culture treated dishes or flasks (section 4.3) using low Ca^{++} KGM with twice-weekly medium changes and were never grown to confluence. These cells were maintained in a humid 37°C/5% CO_2 incubator and all experiments were performed using cultures between passages 1 and 4. Adherent keratinocytes were removed from the culture susbtrate (for example, when subculturing) as follows:

Firstly, the medium was aspirated off the cells and the monolayer was rinsed with 1mM EDTA in PBS to disrupt cell-cell adhesion and to remove any dead cells. The cells were then incubated in trypsin solution (section 4.11.1) at 37°C with occasional gentle agitation until the cells had detached. Trypsin inhibitor solution (section 4.12.1) was then added (twice the volume of trypsin solution) and the cell suspension was pipetted gently to disaggregate the cells. The cells were then pelleted and washed with fresh low Ca^{++} KGM, resuspended in low Ca^{++} KGM, counted and plated at a density of 5×10^3 cells/cm^2.

5.1.3 Keratinocyte cryopreservation and recovery

Cryopreserved human keratinocyte stocks were prepared from freshly isolated keratinocytes as follows:

Keratinocytes prepared from human tissue were allowed to proliferate in low Ca^{++} KGM until they reached 70-80% confluence. They were then trypsinised to remove them from the culture susbstrate but instead of using trypsin inhibitor as described in section 5.1.2, the trypsin was neutralised with serum-containing 10C medium (section 4.11.1). The cells were then rinsed with 10C medium and counted before resuspending in freezing medium (10C + 10% DMSO) at 1×10^6 cells/ml. This cell suspension was transferred into cryotubes (1ml per vial) which were then wrapped in cotton wool and placed in a -70°C freezer overnight. The following day the cryotubes were immersed in liquid nitrogen for long-term storage.

For recovery from storage in liquid nitrogen, cryotubes were rapidly immersed in a large volume of water at approximately 37°C to thaw the cells. The cell suspension was then immediately made up to a volume of 10ml with low Ca^{++} KGM and the cells were pelleted, rinsed with KGM and then resuspended in low Ca^{++} KGM before plating at an initial density of 1×10^6 cells per 9cm dish.

5.2 Cell viability assays

Cell viability assays, to determine the cytotoxicity of the Src-family kinase inhbitor PD162531 (section 4.4) were performed on adherent keratinocytes growing in 96-well microtitre plates using the Promega CellTiter kit. The cells were grown in low Ca^{++} KGM from an initial density of 3×10^3 cells per well and were then incubated in low Ca^{++} KGM containing either PD162531 at concentrations between 0.2 and 20µM, or DMSO (the vehicle for PD162531) at concentrations between 0.002% and 0.2% in a total volume of 100µl per well. All conditions were replicated in triplicate. After 4 days incubation, with daily replacement of medium and Src inhibitor or DMSO, 15µl of dye solution (CellTiter kit) was added to each well and the plate was returned to a 37°C/5% CO_2 incubator for 4 hours. At the end of this incubation, 100µl of solubilisation/stop solution was added to each well and the plate was then sealed and

incubated at room temperature overnight. The following day, the absorbance of each well at 570nm was measured using a plate reader (Molecular Dynamics Emax).

5.3 Flow cytometry

The cell cycle profiles of subconfluent adherent keratinocytes grown in either low Ca^{++} KGM or high Ca^{++} KGM for up to 4 days were determined by flow cytometric analysis of propidium iodide stained cells. Keratinocytes were grown in 75cm^2 flasks in a 37°C/5% CO_2 incubator with daily medium replacement to ensure optimal growth conditions and flasks were shifted to 1mM Ca^{++} at daily intervals to give different incubation times in high extracellular Ca^{++}. The medium was aspirated from the flasks which were then rinsed with warm 1mM EDTA in PBS to disrupt cell-cell adhesion and to remove detached cells. The adherent cells were incubated in 0.25% trypsin solution (4.11.1) for 5 minutes at 37°C and the trypsin was then inhibited by the addition of 10% FCS in PBS. After pipetting gently to disaggregate the cells, they were pelleted and washed with 10ml ice-cold PBS, pelleted again and resuspended in 1ml ice-cold PBS. The cells were then fixed by adding 9ml of 70% ethanol (v/v) whilst mixing gently followed by incubation on ice for 1 hour. Once fixed, the cells were pelleted and after aspirating the ethanol, resuspended in 2.0ml PBS containing 250μg/ml pre-boiled RNAse A, 10μg/ml propidium iodide and 0.2% (w/v) Tween 20. The cells were then incubated at room temperature for 30 minutes before straining through a nylon mesh (70μm pore size) and analysing (Beckton Dickinson FACScan).

5.4 Preparation of protein extracts from adherent keratinocytes

Adherent keratinocytes were lysed in RIPA or CSK buffer (section 4.11.2) to extract cellular proteins as follows:

The flasks or dishes were transferred directly from the 37°C incubator onto ice and were rinsed twice with ice-cold PBS. The culture vessels were then inclined and allowed to drain for 10 minutes to minimise the amount of residual PBS on the cells.

Ice-cold lysis buffer was then pipetted onto the cells (1ml per 9cm plate) which were allowed to lyse for 10 minutes on ice. At the end of this time, the cells were scraped off the tissue culture plastic using a disposable cell scraper and the resulting suspension was transferred into microcentrifuge tubes. The lysates were then cleared of insoluble cell debris by centrifugation at 14,000g at 4°C for 15 minutes. The total protein concentration of the lysates was estimated using the BCA protein assay kit according to the manufacturers directions. The aborbance of the samples was then measured at 562nm using a Beckman DU 650 spectrophotometer. Experimental values were compared to a standard curve (obtained using BSA) and the total protein concentration in each sample calculated.

5.5 Preparation of fractionated lysates from adherent keratinocytes (Triton-soluble and insoluble proteins)

For some experiments, lysates were prepared by a 2-stage process using a buffer containing only non-ionic detergent first (CSK buffer, section 4.11.2) followed by SDS-containing RIPA buffer. The cells were initially treated as described in section 5.4, using CSK buffer. After the lysates had been cleared by centrifugation, the insoluble pellets were resuspended in RIPA buffer and sonicated (MSE soniprep 150) briefly on ice to disaggregate the material. The resulting suspension was then centrifuged as described in section 5.4 to pellet the SDS-insoluble material which was subsequently discarded. Thus, the supernatant from the first centrifugation contained the Triton-soluble proteins (TXs) and the supernatant from the second centrifugation contained the Triton-insoluble (but SDS-soluble) proteins (TXi).

5.6 Protein electrophoresis

Lysates prepared in either CSK or RIPA buffer as described in sections 5.4 and 5.5 were standardised for total protein content before adding 3× sample buffer (2 parts lysate to 1 part buffer, section 4.11.1). The lysates were then boiled for 5 minutes and allowed to cool before loading onto SDS-polyacrylamide gels for electrophoresis.

Protein samples were separated on discontinuous 2mm SDS-polyacrylamide gels comprising a short stacking gel for the initial stage of the electrophoresis and a longer resolving gel to separate the proteins. The resolving gels contained either 7.5% acrylamide where the protein of interest was larger than 60kDa, or 10% acrylamide for the smaller proteins. The detailed composition of these gels is given in section 4.11.2. Gels were typically loaded with 20-100μg total protein per lane and were run at either 180V for 3-4 hours or 30V overnight under constant-voltage conditions until the dye-front had reached the bottom of the gel. All gels were run with molecular weight markers (section 4.8)

5.7 Immunoblotting

5.7.1 Transferring proteins onto nitrocellulose

After electophoresis, proteins were transferred onto nitrocellulose membrane by electrophoretic semi-dry blotting essentially as described in Harlow and Lane, 1988. Briefly, the SDS-PAGE gel, nitrocellulose membrane and 12 pieces of 3MM paper were soaked in transfer buffer (section 4.11.2) for 10 minutes and were then arranged between the blotter electrodes with 6 sheets of 3MM paper on either side of the gel/membrane sandwich oriented with the membrane on the cathode side of the gel. After gently excluding air bubbles from the paper/gel/membrane stack, the proteins were transferred for 1 hour at a maximum current of 200mA and a maximum voltage of 20V for a gel of $140cm^2$.

5.7.2 Antibody incubations and detection by ECL

Following the transfer, the nitrocellulose membrane was stained briefly in Ponceau S solution (section 4.8) then destained in wash buffer (section 4.11.2) to check for even transfer of proteins. Blots were then blocked in the appropriate blocking solution (either 6% (w/v) non-fat milk or 3% (w/v) BSA in wash buffer) for 1 hour at room temperature on a rotary shaker. After blocking, the blots were probed with primary antibody diluted in blocking solution for either 1 hour at room temperature or overnight at 4°C. The primary antibodies, dilutions and blocking solutions are summarised in table 1.

After incubation in primary antibody, the blots were washed 3 times in wash buffer (15 minutes per wash) and were then incubated in horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. Secondary antibodies are summarised in table 2. After washing as descibed above, the blots were drained and incubated in fresh ECL reagent for 1 minute with gentle agitation and were then wrapped in Saran-Wrap. The blots were placed in contact with X-ray film for a period of time sufficient to detect the light produced by the ECL reaction and the film was then processed in a Kodak automated processor.

5.7.3 Stripping immunoblots to remove bound antisera

Nitrocellulose blots which had been previously incubated in antisera were treated as follows to remove these bound antibodies prior to incubation with a different antiserum. The blot was firstly washed with immunoblot wash buffer (4.11.2) to remove spent ECL reagent and was then incubated in immunoblot stripping buffer (4.11.2) for 30 minutes at room temperature on a gyratory shaker. At the end of this incubation, the blot was washed again and then blocked and incubated with the desired antibodies as described in section 5.7.2.

5.8 Immunoprecipitation of extracted proteins

Lysates prepared in RIPA or CSK buffer (4.11.2) were standardised for total protein content (0.25-1.0mg) before preclearing with either 0.5μg normal mouse IgG or normal rabbit serum depending on the species of origin of the immunoprecipitating antiserum, for 1 hour at 4°C. The IgG complexes were then collected by incubating for 1 hour at

Tables 1 and 2

Table 1
Primary antisera used for immunoblotting

Protein recognised	Antibody name	Species/type	Dilution	Blocking solution
E-cadherin	Clone #36	mouse monoclonal	1:2000	milk
P-cadherin	Clone #56	mouse monoclonal	1:500	BSA
β-catenin	-	rabbit polyclonal	1:2000	milk
β_1-integrin	mAb 13	rat monoclonal	1:1000	BSA
c-Src	mAb 327	mouse monoclonal	1:1000	BSA
Fyn	Fyn (3)	rabbit polyclonal	1:500	BSA
c-Yes	Clone #1	mouse monoclonal	1:1000	milk
$p120^{ctn}$	Clone #98	mouse monoclonal	1:1000	milk
Involucrin	SY5	mouse monoclonal	1:1500	milk
Phosphotyrosine	PY20	mouse monoclonal	1:1000	BSA
K14	LL001	mouse monoclonal	1:1000	BSA
α-Tubulin	DM 1A	mouse monoclonal	1:10000	BSA

LL001 was a generous gift from Professor E.B. Lane, University of Dundee.

Table 2
Secondary antisera used for immunoblotting

Antibody recognised	Species	Dilution
mouse IgG	sheep	1:3000
rabbit IgG	donkey	1:5000
rat IgG	rabbit	1:5000

4°C with 50μl of a 50% slurry of protein-A sepharose beads (coated with rabbit anti-mouse IgG for normal mouse IgG or left uncoated for rabbit serum). The immunoprecipitating IgG (typically 0.5μg) was added to the precleared lysate which was then was incubated overnight at 4°C before collecting the IgG complexes with coated or uncoated protein-A sepharose beads as described above. The collected immune complexes were washed 5 times in RIPA lysis buffer before resuspending in 20μl of 3× sample buffer prior to electrophoresis and blotting as detailed in sections 5.6 and 5.7.

5.9 *IN VITRO* TYROSINE KINASE ASSAYS

Assays to determine the kinase activity of c-Src extracted from keratinocytes grown under different conditions were performed using a synthetic poly(Glu,Tyr) peptide as an exogenous substrate. Adherent keratinocytes were lysed in CSK buffer and the lysates were cleared of insoluble debris by centrifugation at 14,000g at 4°C for 15 minutes prior to determining the total protein concentration as detailed in section 5.4. c-Src, c-Yes or Fyn was then immunoprecipitated from lysate containing 250μg total protein (c-Src) or 1mg total protein (Fyn and c-Yes) as described in section 5.8 using 0.5μg of antiserum per sample. The immunoprecipitates were then washed 5 times in 0.5ml CSK buffer followed by one wash in kinase buffer (section 4.12.2) and the pellets were then resuspended in 25μl kinase buffer for use in the kinase assay. Each 20μl kinase assay reaction contained the following:

2mg/ml substrate peptide
1μM ATP
5μCi [γ-^{32}P]ATP (specific activity 3000Ci/mmol)
10μl resuspended immunoprecipitate
1.5μl kinase buffer

The reactions were started by adding the resuspended immune complex and were then incubated at 30°C for 7.5 minutes before stopping the reaction by the addition of excess unlabelled ATP and chilling on ice. The reaction mixtures were then spotted onto p81

paper squares which were subsequently immersed in a large volume of 10% TCA and washed 3 times with fresh 10% TCA (5 minutes per wash). The paper squares were transferred to scintillation vials to which 5ml of Ecoscint was added and the radioactivity present in each vial was measured using a Beckman LS 60001C liquid scintillation counter. Control reactions without substrate peptide were also performed.

5.10 Cornified envelope counts

Adherent keratinocytes were trypsinised (section 5.1.2) and were then counted, pelleted and resuspended in high Ca^{++} KGM containing ionomycin at a final concentration of 10μM. The cells were then incubated at 37°C in sealed tubes gassed with a 5% CO_2 mixture for a duration of 4 hours. Control cells were treated with DMSO (the vehicle for ionomycin) at a final concentration of 0.01% (w/v) under the same conditions. The cells (1×10^5 cells/ml) were then boiled for 10 minutes in 5% SDS, 1% 2-mercaptoethanol to solublise the cellular proteins, leaving intact envelopes (Sun and Green, 1976) which were visualised microscopically and counted using a haemacytometer. The proportion of cells that had produced envelopes was then calculated.

5.11 Immunofluorescence microscopy

Keratinocytes were grown on 8-well chamber slides from an initial density of 5×10^3 cells per well in low Ca^{++} KGM. Where appropriate, some wells were shifted to high Ca^{++} KGM for a suitable interval. The cells were then washed with ice-cold PBS and fixed on ice for 20 minutes in absolute methanol chilled to -20°C. The slides were washed twice in KRH buffer (section 4.11.3) before blocking with 10% foetal calf serum diluted in KRH buffer (blocking buffer). After blocking, the cells were incubated for 1 hour at room temperature in primary antibody (table 3) diluted in blocking buffer and were subsequently washed for 3 periods of 10 minutes in KRH buffer. The cells were then incubated in fluorochrome-conjugated secondary antibody (summarised in table 4).

Tables 3 and 4

Table 3

Primary antisera used for immunofluorescence

Protein recognised	Antibody	Dilution
actin	AC-40	1:100
c-Src	mAb 327 ascites[1]	1:100
Fyn	Fyn(3)	1:100
c-Yes	Clone #1	1:100
E-cadherin	Clone #36	1:250
E-cadherin	DECMA-1	1:250
P-cadherin	mAb 6A9[2]	1:25
$p120^{ctn}$	Clone #98	1:100
β_1-integrin	mAb 13	1:100
α-Tubulin	DM 1A	1:1000

[1]mAb 327 ascites was a generous gift from Dr. S. Simon, Salk Institute.

[2]mAb 6A9 was a generous gift from Dr. M. Wheelock, University of Toledo, Ohio.

Table 4

Secondary antisera used for immunofluoresence

Antibody	Dilution
Anti-rat/FITC conjugate	1:125
Anti-rabbit/FITC conjugate	1:40
Anti-mouse/FITC conjugate	1:100

diluted in blocking buffer, for 1 hour at room temperature in a darkened humid box before washing again. When nuclei were stained with DAPI, it was added to the final wash at a concentration of 0.1µg/ml. The plastic chambers and sealing gasket were removed from the slides and a drop of Vectashield mounting medium was applied to each area of cells. A glass coverslip was then placed over the cells and sealed in place using clear nail varnish and the slides were viewed using either a Zeiss fluorescence microscope or a confocal fluorescence microscope (Model MRC 600, BioRad, Hercules, CA).

5.12 IMMUNOPEROXIDASE STAINING

Immunoperoxidase staining of adherent keratinocytes was performed using the Vectastain kit. Cells were grown on chamber slides and fixed in the same way as for immunofluorescence (section 5.11). After fixation, the cells were washed twice with immunoperoxidase wash buffer (section 4.12.3) before blocking in 10% goat serum diluted in loading buffer (section 4.12.3) for 1 hour at room temperature in a humid box. After blocking, the cells were incubated overnight at 4°C in primary antibody diluted in loading buffer. The anti-involucrin and anti-transglutaminase antisera (SY5 and B C1 respectively) were diluted 1:1000 whilst the anti-filaggrin antiserum (BT-576) was diluted 1:500.

After incubating in primary antibody, the cells were washed (3 × 10 minutes) in wash buffer and then incubated for 1 hour at room temperature in the biotinylated secondary antibody (anti-mouse) provided with the kit, diluted 1:200 in loading buffer. At the end of the incubation in secondary antibody, the cells were washed as before, then incubated in biotinylated peroxidase that had been allowed to complex with avidin (1 drop of kit reagent A and one drop of reagent B in 5ml of loading buffer). This incubation was for 1 hour at room temperature and was followed by a further 3 × 10 minute washes in wash buffer. The cells were then incubated in substrate solution (4.12.3) for 7½ minutes at room temperature before rinsing thoroughly with tap water. Coverslips were then mounted using mounting solution (section 4.11.3) and were sealed in place with clear nail varnish. The slides were viewed using a Nikon Diaphot phase-contrast microscope.

5.13 Cell surface cadherin and integrin clustering

Cells were seeded onto 8-well chamber slides in low Ca^{++} KGM at an initial density of 5×10^3 cells per well and were subsequently grown for 1-2 days before clustering E-cadherin in low Ca^{++} as follows:

The cells were firstly chilled by placing the slides on ice for 10 minutes and the medium was then replaced with antibody (anti-E-cadherin (DECMA-1) or anti-β_1-integrin rat mAb, which had been depleted of sodium azide, section 5.15) diluted in ice-cold low Ca^{++} KGM. The antibody dilutions varied between batches but were typically 1:10 for DECMA-1 and 1:50 for mAb 13. The cells were incubated in the diluted antibody on ice for 30 minutes and were then washed extensively with ice-cold KGM to remove unbound antibody. Diluted second antibody (goat anti-rat IgG at the same dilution as the first antibody) was then added in ice-cold KGM and the cells were immediately returned to a 37°C/5% CO_2 incubator. Four hours later the cells were washed with warm KGM to remove excess second antibody. When the cells were to be visualised for clustered cadherin or integrin on the cell surface, they were fixed at this stage. However, when the purpose of the experiment was to investigate differentiation-specific protein expression, the cells were then incubated in low Ca^{++} KGM (without antibodies) for a further 4 days before fixing and staining for the relevant proteins as described in section 5.12.

5.14 Removal of sodium azide from commercial antisera

Antisera used for E-cadherin and β_1-integrin (DECMA-1 and mAb13 respectively) clustering were treated using Centricon-10 concentrators to substantially deplete the preparation of sodium azide as follows:

The surfaces of the concentrator which would contact the antiserum during the process were incubated in 1% (w/v) BSA in PBS for 30 minutes at room temperature to block any protein binding sites. The concentrator was then rinsed thoroughly with sterile PBS and filled with antiserum (up to 1.5mg) diluted to a total volume of 2ml with ice-cold sterile PBS. The concentrators were then centrifuged at 4000g in a refrigerated

centrifuge at 4°C for 90 minutes using a fixed angle rotor, which was sufficient to concentrate the antiserum solution to a volume of approximately 200μl. This was then diluted to a total volume of 2ml with sterile PBS and the centrifugation process repeated to give 200μl of concentrated antiserum containing approximately 1% of the original sodium azide concentration.

5.15 CADHERIN BLOCKING

Keratinocytes were cultured in 8-well chamber slides as described in section 5.13 and were treated with synthetic decapeptides to interfere with cadherin function. The blocking (LRAHAVDVNG-amide) and control peptides (VIPPINLPEN-amide) were identical to those described by Blaschuk *et al.,* (1990) which interfere with the functions of both E- and N-cadherin. These peptides were diluted in low Ca^{++} KGM to give a final peptide concentration of 0.2mg/ml and the cells were incubated in these diluted peptides for 4 hours at 37°C/5% CO_2. After this time, $CaCl_2$ solution was added to some wells giving a final Ca^{++} concentration of 1mM. The cells were incubated in the presence of blocking or control peptide in high or low extracellular Ca^{++} for a further 4 days before fixing and staining for differentiation-specific proteins as described in section 5.13. The medium in all wells containing diluted peptide was replaced daily.

RESULTS AND DISCUSSION

CHAPTER 6

KERATINOCYTE BEHAVIOUR IN THE SERUM-FREE SYSTEM

6.1 PURPOSE

The original objective of this study was to determine the origin of the Ca^{++}-induced signal that induces the keratinocyte differentiation program and to investigate how this might utilise the members of the Src family of tyrosine kinases which are implicated in keratinocyte differentiation (Calautti *et al.*, 1995; Zhao *et al.*, 1992 and 1993). This chapter describes a detailed characterisation of the responses of keratinocytes prepared and maintained in the serum-free system to changes in the extracellular Ca^{++} concentration. I chose a low extracellular Ca^{++} concentration of 0.03mM for routine keratinocyte propagation as this is the lowest concentration at which keratinocytes can be maintained (Boyce and Ham, 1983). A high extracellular Ca^{++} concentration of 1mM was chosen to induce differentiation as this is the concentration routinely used by other investigators.

6.2 CALCIUM INDUCES THE EXPRESSION OF DIFFERENTIATION-SPECIFIC PROTEINS

6.2.1 Calcium induces involucrin, transglutaminase and filaggrin expression

A. Immunoperoxidase staining

In order to investigate the expression of differentiation-specific proteins, early passage keratinocytes were grown on 8-well chamber slides in 0.03mM Ca^{++} (LKGM). Twenty-four hours later, some wells were switched to high Ca^{++} KGM (HKGM). At daily intervals, additional cells were switched from LKGM to HKGM such that cells had been incubated in HKGM for a duration of 0, 1, 2 or 4 days by the time of fixation. This strategy of switching to 1mM Ca^{++} at intervals was chosen to permit all the cells to be fixed simultaneously, thus eliminating the potential effects of cell density on differentiation. After fixing, the cells were stained for involucrin, keratinocyte

transglutaminase (TGk) or filaggrin using the immunoperoxidase method (section 5.12) to assess the effects of Ca^{++} on the expression of these differentiation-specific proteins (Figures 6, 7 and 8).

Keratinocytes grown in KGM containing 0.03mM Ca^{++} (LKGM) showed low levels of expression of all three proteins (Figures 6 *a*, 7 *a*, and 8 *a*). Brown staining for both involucrin and TGk was visible in occasional large, spontaneously differentiated cells but no filaggrin staining was visible in keratinocytes grown under these conditions. One day after switching to HKGM (Figures 6 *b*, 7 *b*, and 8 *b*), no appreciable increase in the number of cells staining for involucrin or filaggrin was apparent but there was an increased number of cells staining for TGk. During this time the arrangement of the cells on the substrate changed dramatically with the cells aggregating into tightly packed groups (see section 6.4) which became multilayered (stratified) after approximately 24 hours incubation in HKGM. The differentiated cells were located in the upper layers of these stratified groups of cells. After 48 hours in HKGM (*c*), significantly more cells stained for both involucrin and TGk but again, no filaggrin staining was visible. Filaggrin-positive cells first became visible four days after switching to HKGM at a time when a large proportion of the culture was also positive for involucrin and TGk. Thus, under these conditions, high extracellular Ca^{++} increases the proportion of adherent cells expressing these three differentiation-specific proteins.

B. Involucrin immunoblotting

Protein extracts prepared from cells grown in LKGM and HKGM for up to 5 days were immunoblotted for involucrin (Figure 9). In cells which were maintained in low extracellular Ca^{++} for the duration of this experiment (Figure 9 *a*), basal levels of involucrin were low (t = 0) and showed a very small increase over time, consistent with a low rate of spontaneous differentiation. The results obtained for cells shifted to HKGM at t = 0 (Figure 9 *b*) showed a marked increase in involucrin expression as early as one day after the switch to HKGM. The involucrin protein levels progressively increased thereafter. Unfortunately, the anti-TGk and anti-filaggrin antisera did not work in immunoblotting.

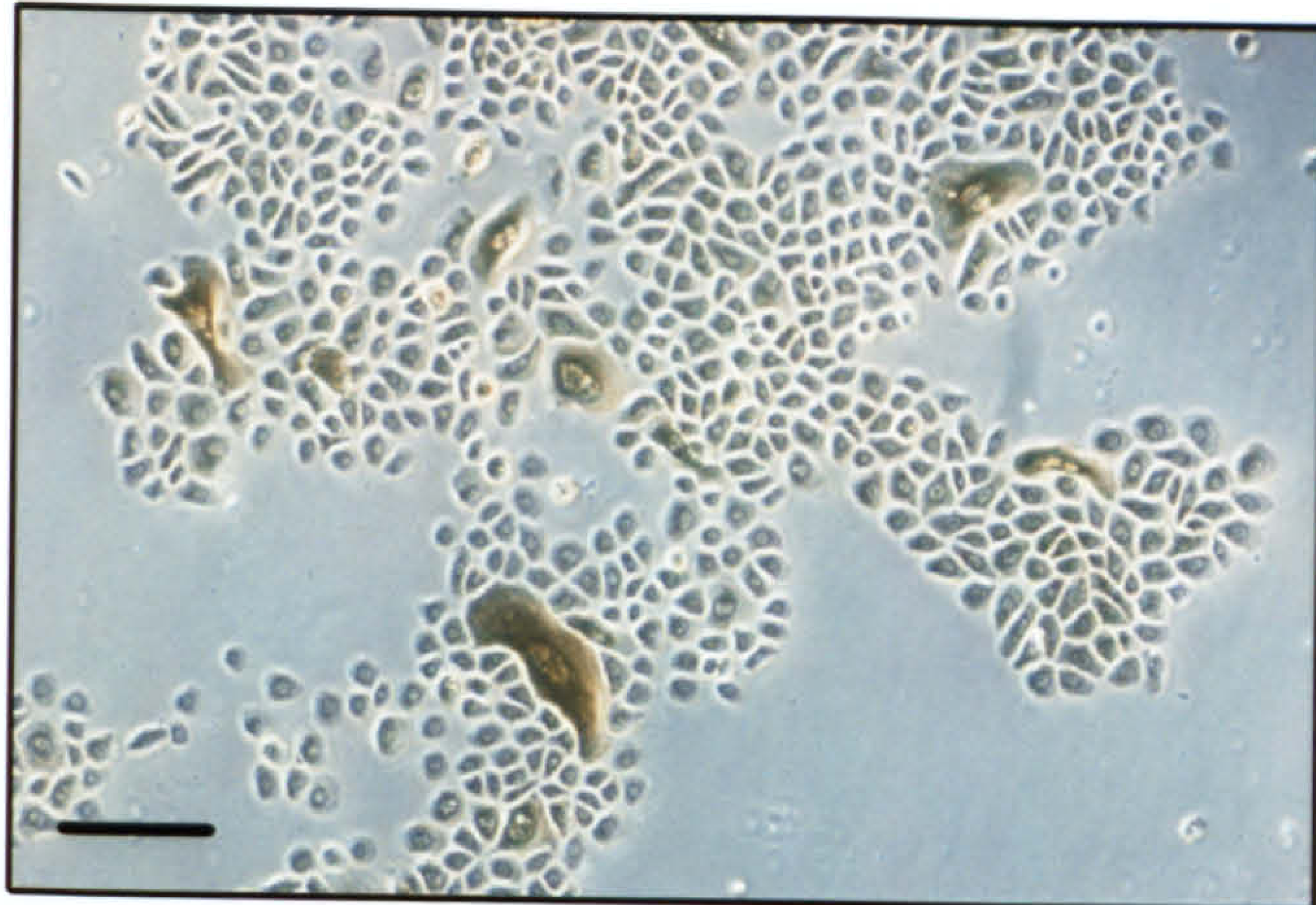
Figure 6

Figure 6
The induction of involucrin expression by high extracellular calcium

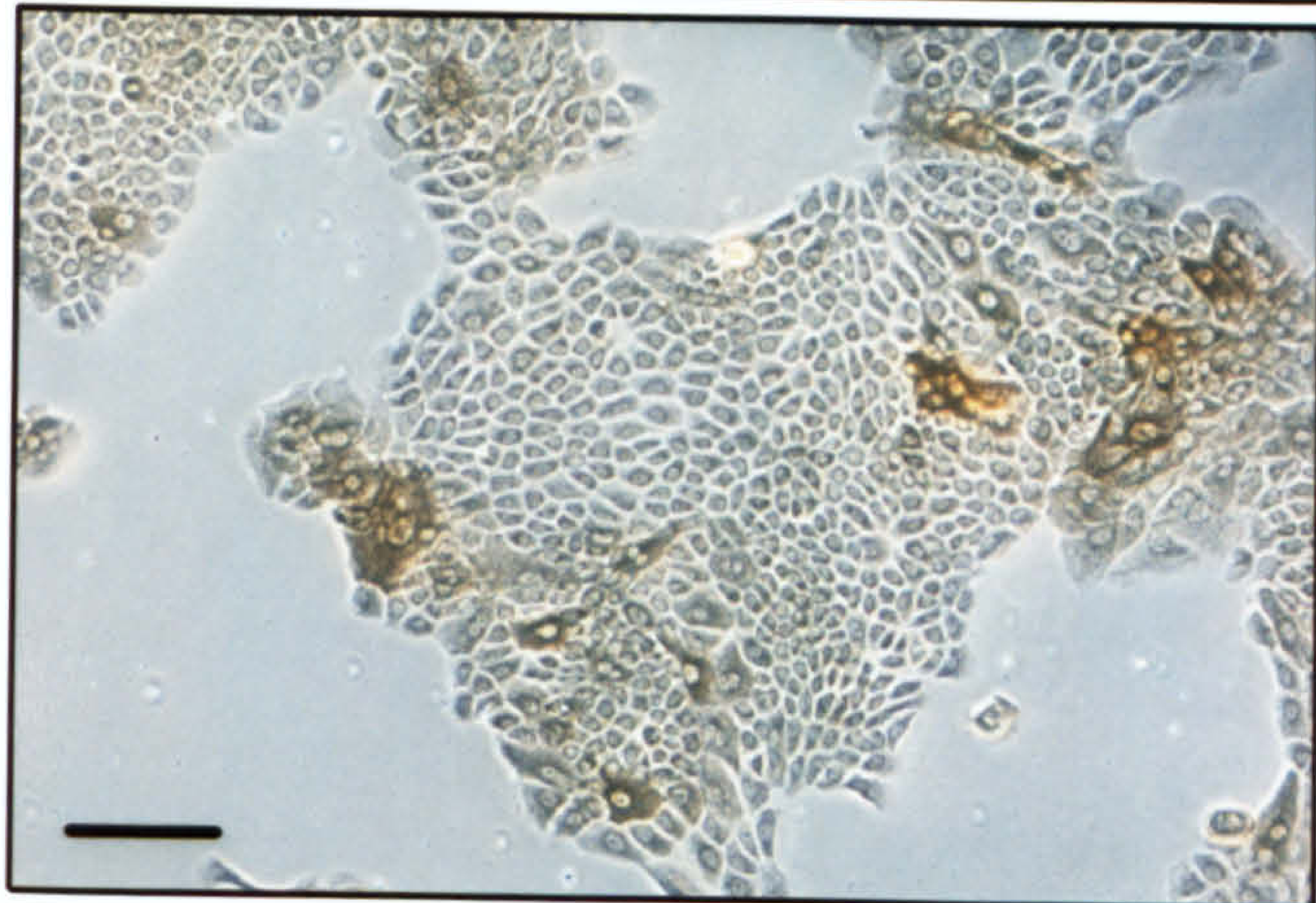
Phase-contrast photomicrographs of adherent keratinocytes stained for the differentiation-specific protein involucrin using the immunoperoxidase method after incubation in 0.03mM extracellular Ca^{++} (*a*) and at 1, 2 and 4 days after shifting to 1mM Ca^{++} (*b, c* and *d* respectively). The antiserum used was anti-involucrin, mAb SY5 diluted 1:1000. Scale bars represent 200μm.

The induction of involucrin expression by high extracellular calcium

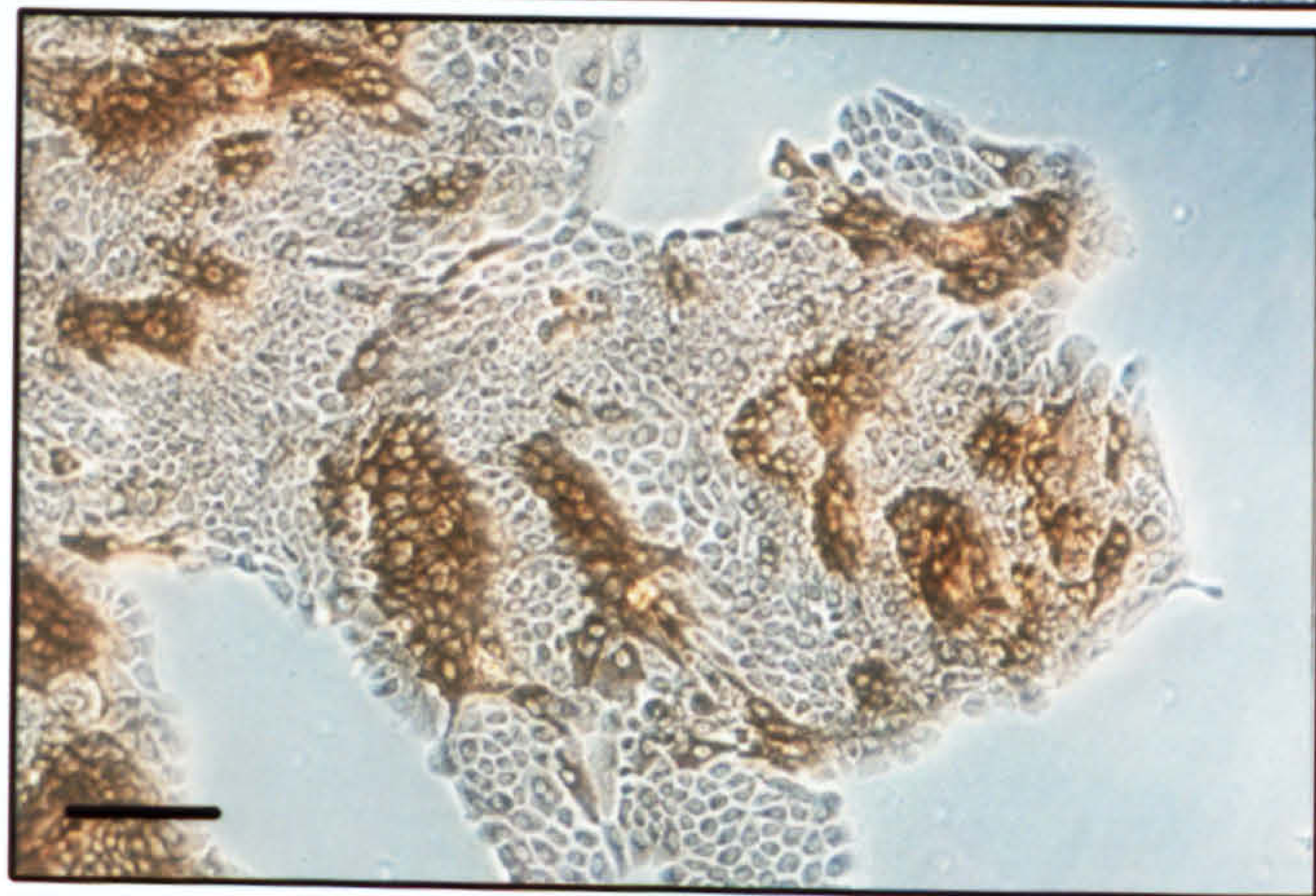
a. low extracellular calcium



b. high extracellular calcium, 1 day



c. high extracellullar calcium, 2 days



d. high extracellular calcium, 4 days

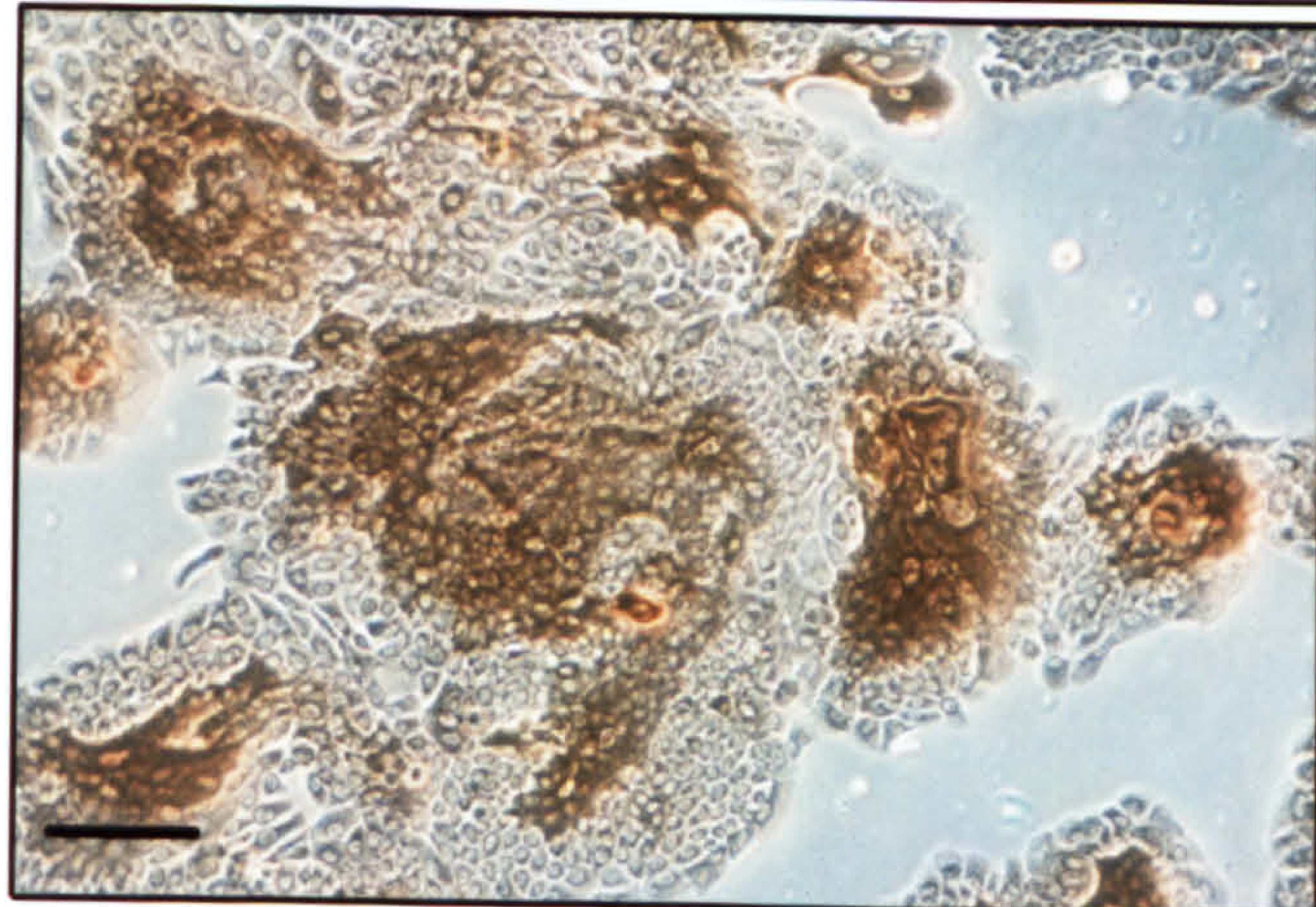


Figure 7

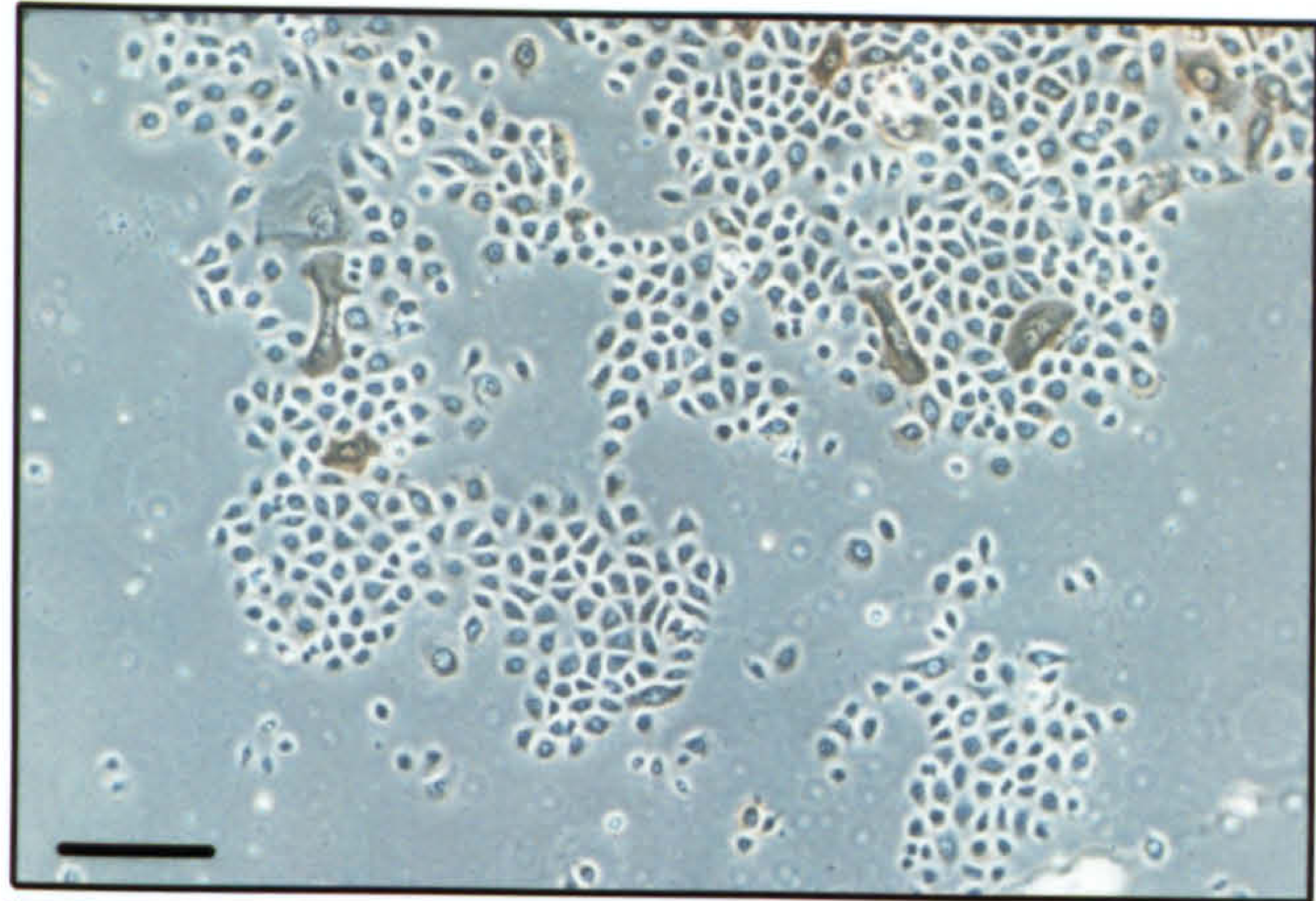
Figure 7

The induction of transglutaminase expression by high extracellular calcium

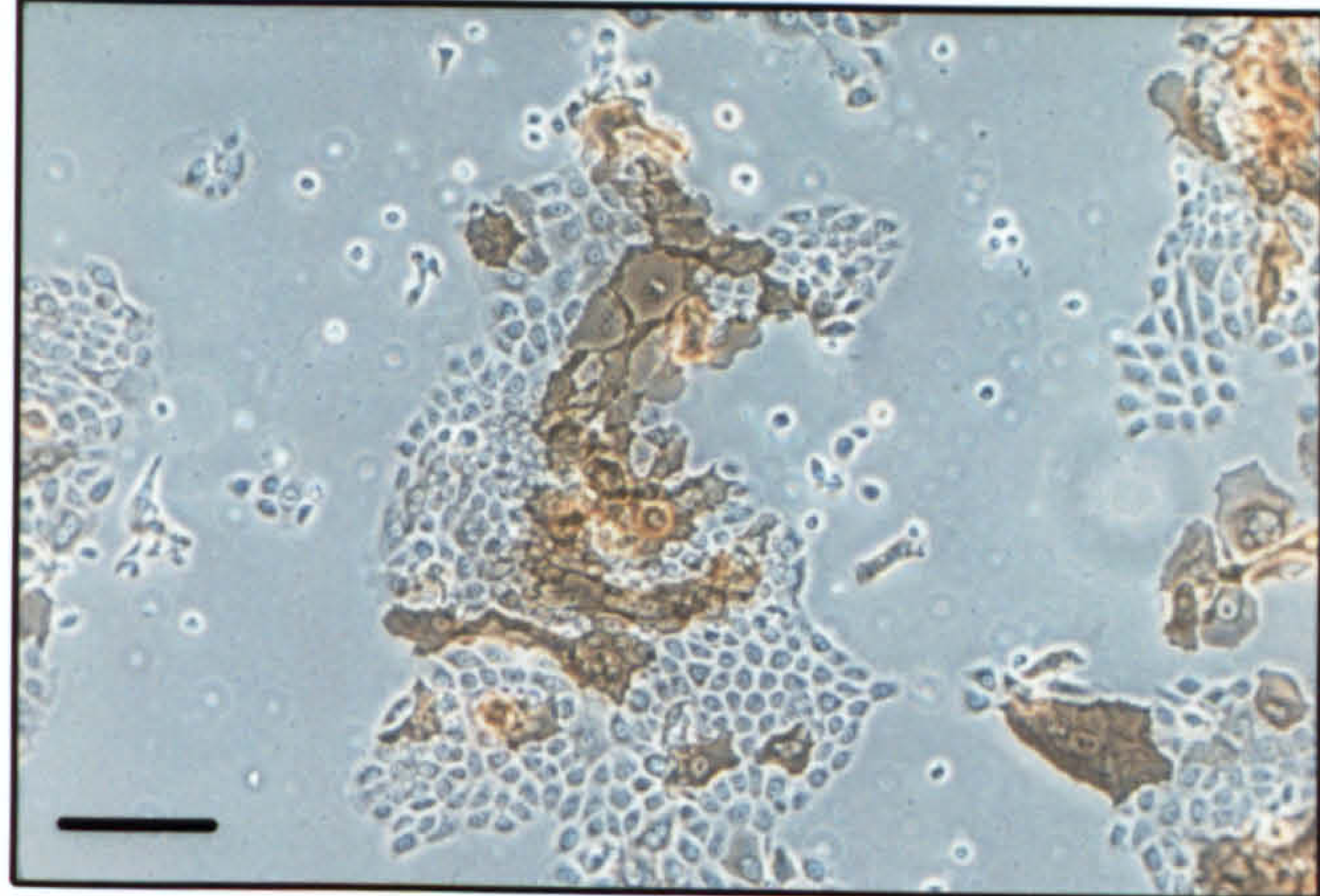
This figure shows phase-contrast photomicrographs of adherent keratinocytes stained for one of the enzymes responsible for assembling the cornified envelope (keratinocyte transglutaminase/TGk). The cells were stained using the immunoperoxidase method after incubation in 0.03mM extracellular Ca^{++} (*a*) and at 1, 2 and 4 days after shifting to 1mM Ca^{++} (*b, c* and *d* respectively). The antiserum used was anti-transglutaminase, mAb B. C1 diluted 1:1000. Scale bars represent 200μm.

The induction of transglutaminase expression by high extracellular calcium

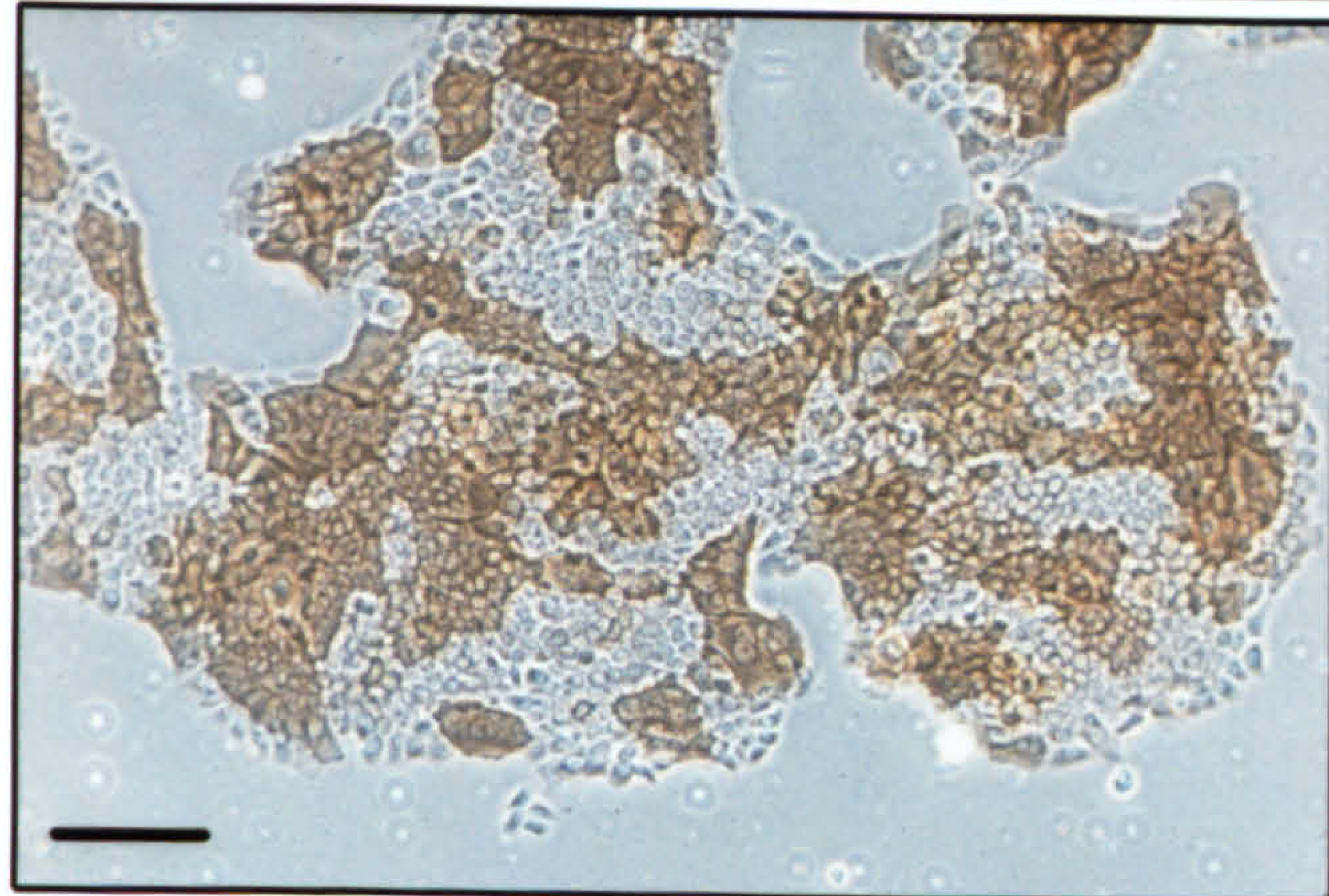
a. low extracellular calcium



b. high extracellular calcium, 1 day



c. high extracellular calcium, 2 days



d. high extracellular calcium, 4 days

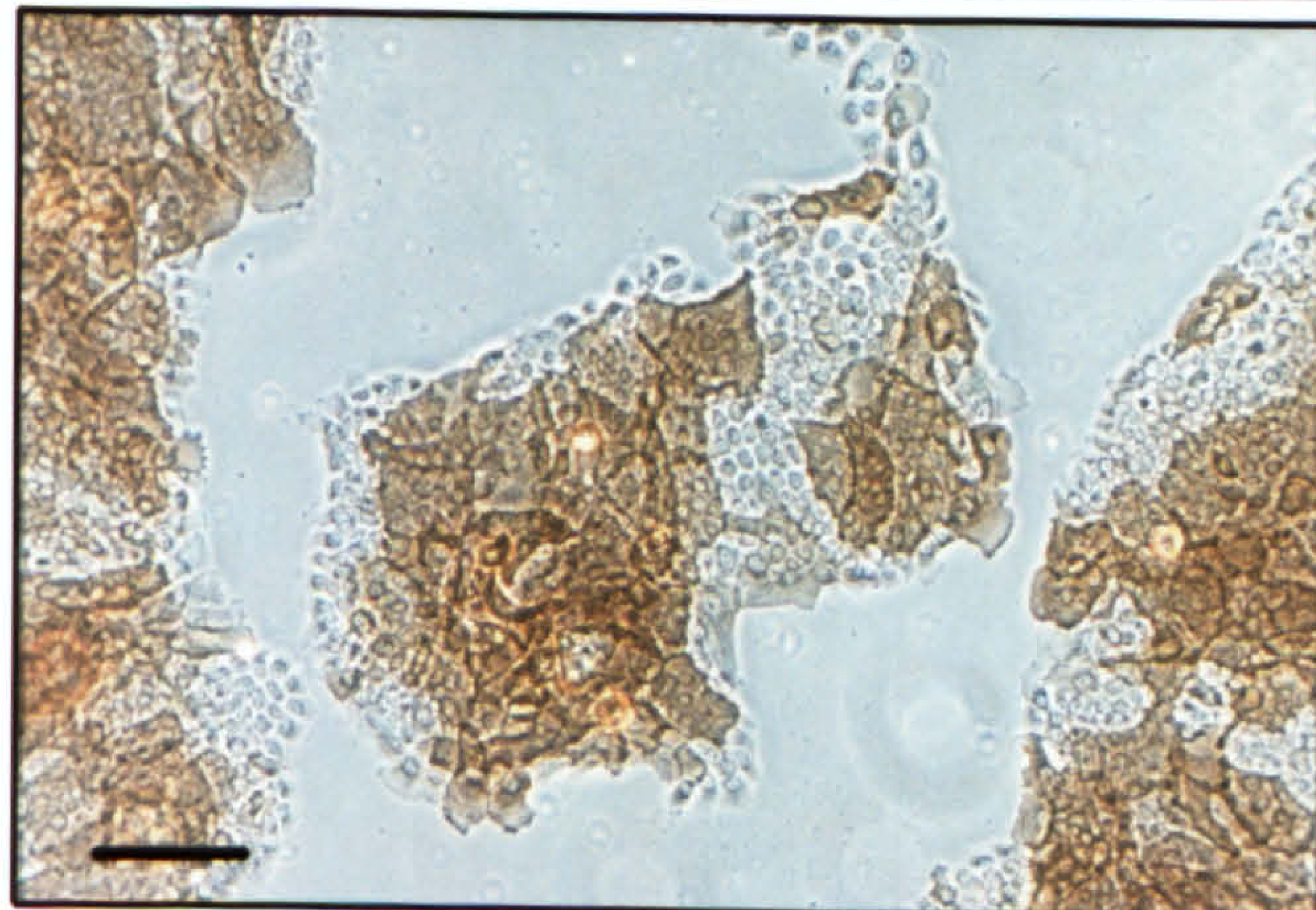


Figure 8

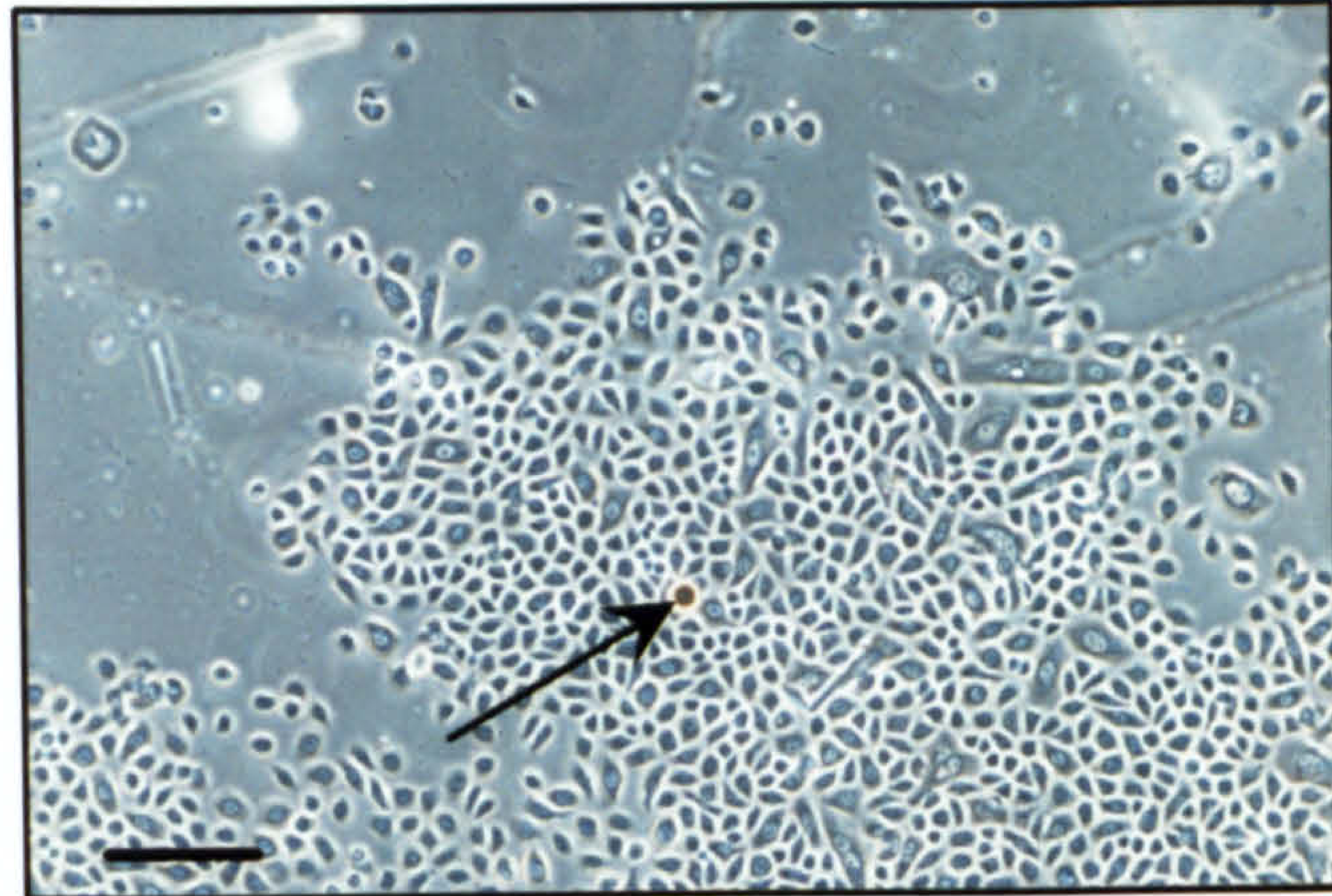
Figure 8

The induction of filaggrin expression by high extracellular calcium.

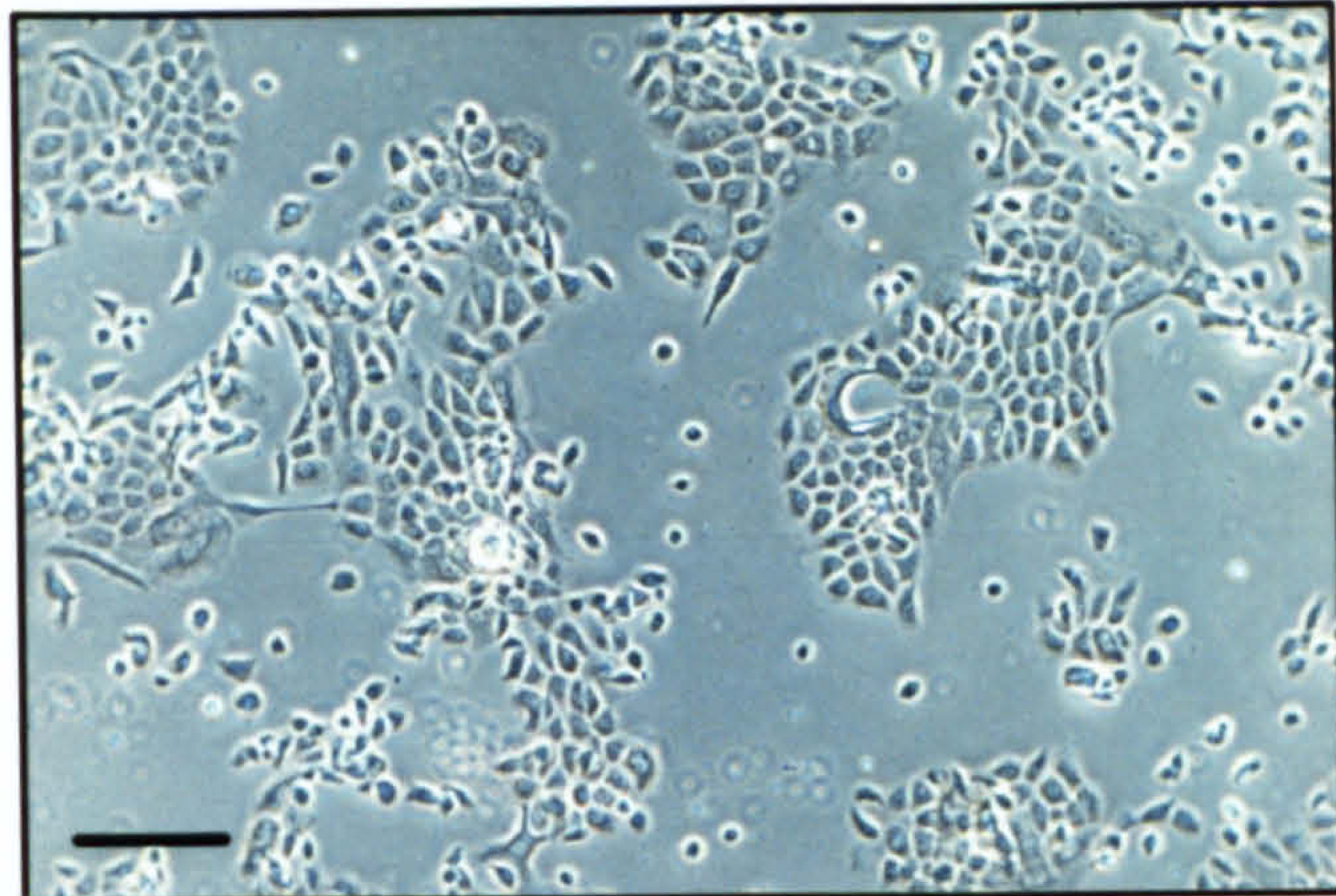
This figure shows phase-contrast photomicrographs of adherent keratinocytes stained for the differentiation-specific protein filaggrin using the immunoperoxidase method. The cells were stained after incubation in 0.03mM extracellular Ca^{++} (*a*) and at 1, 2 and 4 days after shifting to 1mM Ca^{++} (*b, c* and *d* respectively). The arrow (*a*) indicates a cell stained for filaggrin. The antiserum used was anti-filaggrin mAb BT-576 diluted 1:500. Scale bars represent 200μm.

The induction of filaggrin expression by high extracellular calcium

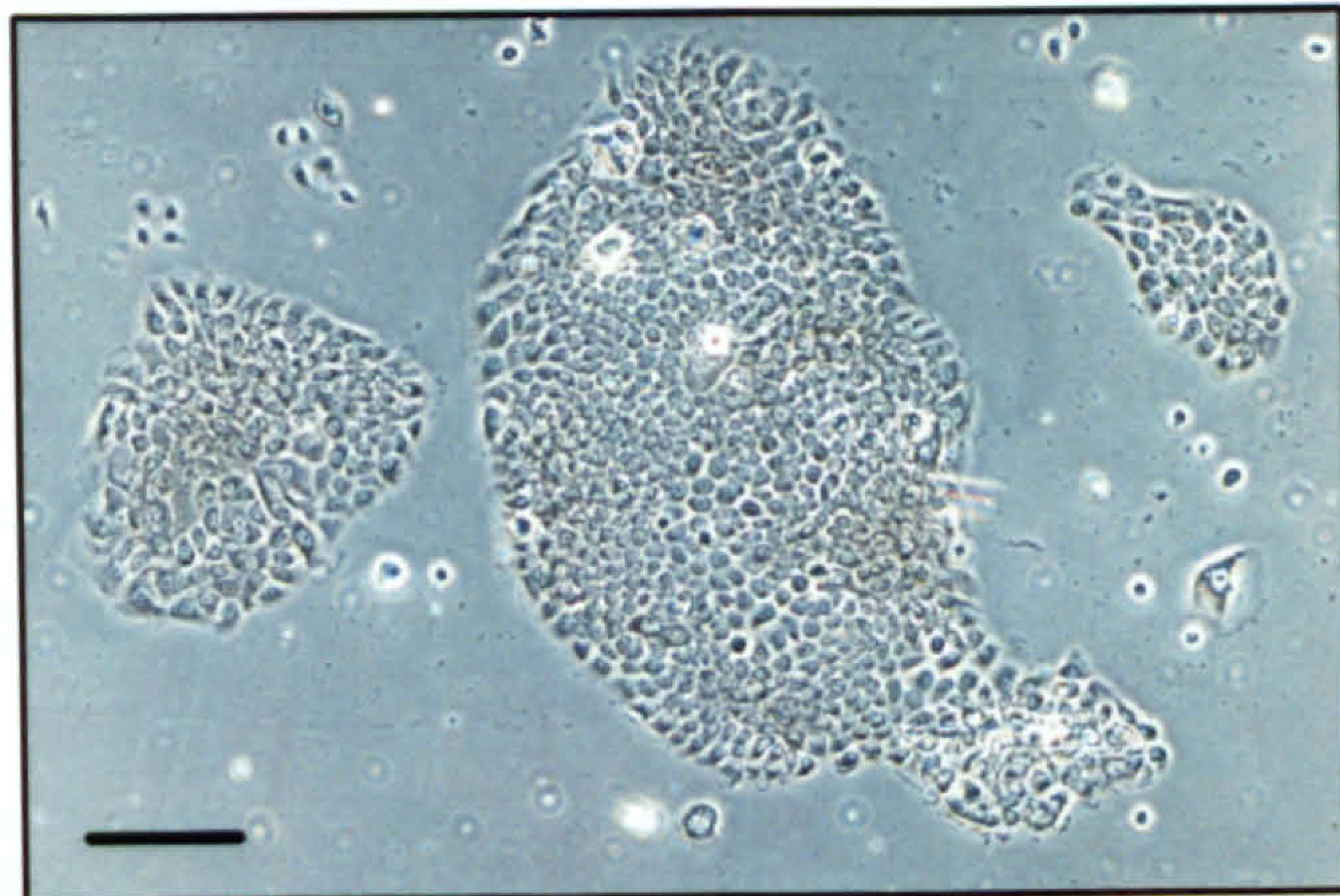
a. low extracellular calcium



b. high extracellular calcium, 1 day



c. high extracellular calcium, 2 days



d. high extracellular calcium, 4 days

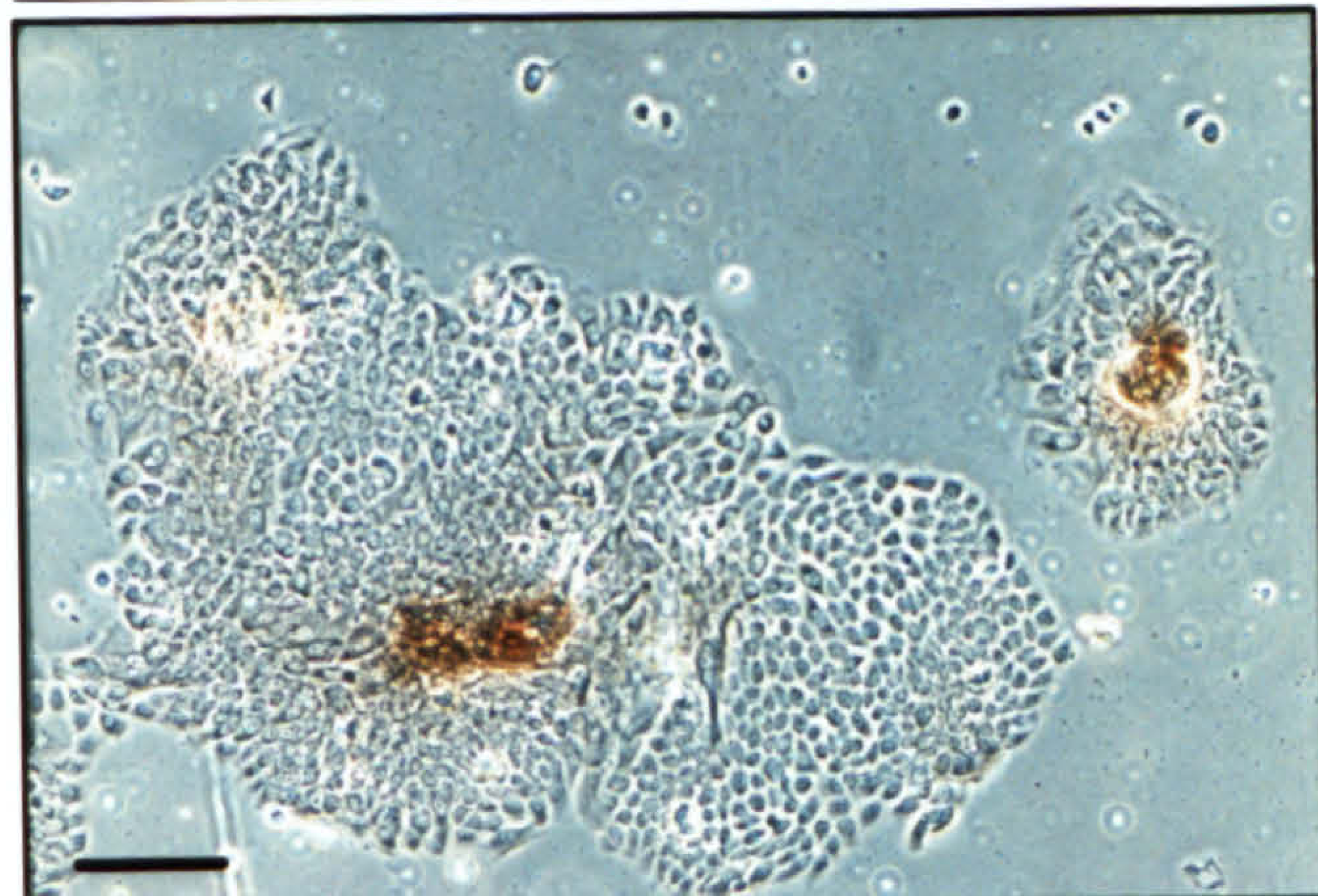


Figure 9

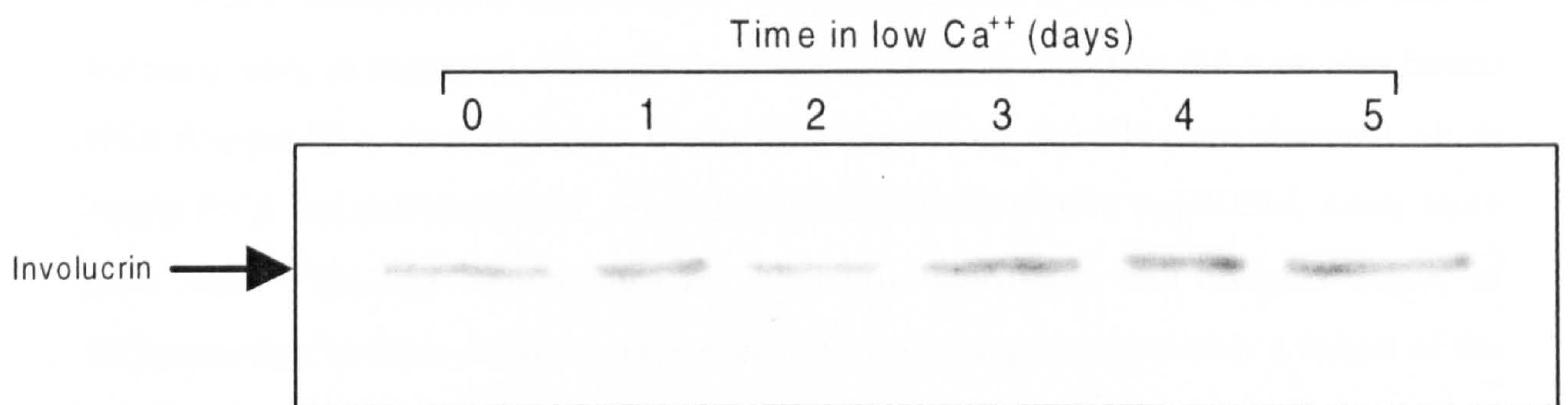
Figure 9

The induction of involucrin expression by high extracellular calcium

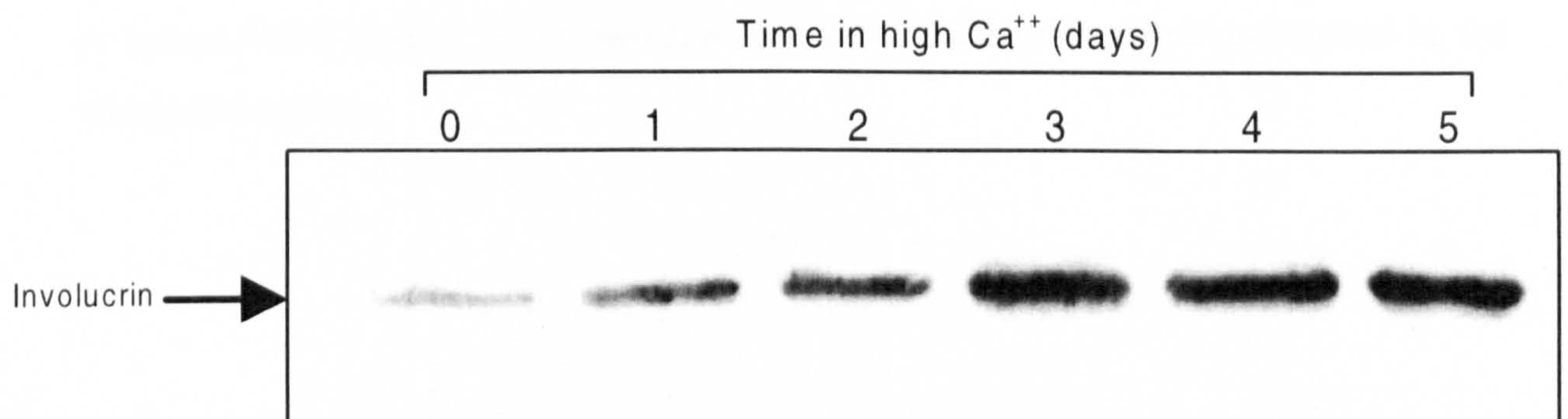
This figure shows two immunoblots for involucrin using lysates prepared in CSK buffer from adherent keratinocytes grown in low (*a*) and high (*b*) extracellular Ca^{++}. Molecular weight markers are not indicated on these two panels as they ran outside the regions of the blots shown. The antiserum used was anti-involucrin, mAb SY5 diluted 1:2000.

The induction of involucrin expression by high extracellular calcium

a. **low extracellular calcium**



b. **high extracellular calcium**



6.2.2 Calcium induces peanut agglutinin binding activity

The induction of another indicator of keratinocyte differentiation by high extracellular Ca^{++} was investigated. Peanut agglutinin (PNA) binding is a feature of keratinocyte differentiation (Ookusa *et al.*, 1983) and it is reported that PNA binds to the transmembrane glycoprotein CD44 in these cells (Hudson *et al.*, 1995). To investigate the induction of PNA binding activity by high extracellular Ca^{++}, keratinocytes were grown in chamber slides as described in section 6.2.1A in either LKGM or in HKGM for a duration of 5 days before staining with anti-involucrin, 4′,6-diamidino-2-phenylindole (DAPI) and fluorescein-labelled peanut agglutinin (Figure 10).

Where subconfluent keratinocytes were maintained in LKGM, few cells stained for involucrin, as expected, although the majority of those cells that did stain also bound PNA (Figure 10 *a*, closed arrow). Under these conditions no cells were observed which bound PNA but not involucrin. Five days after switching cells to HKGM, many more cells bound PNA and were stained for involucrin, consistent with a higher degree of differentiation in these cultures. However, it was also apparent that only a subset of the involucrin positive cells bound PNA, appearing yellow in the merged immunofluorescence images (Figure 10 *b*, open arrow). The nuclei of these differentiated cells are not readily visible in this image as the blue staining is masked by the stronger staining for involucrin and peanut agglutinin. In addition, the undifferentiated keratinocytes adjacent to the substrate are visible around the periphery of these stratified groups of cells. Thus, high extracellular Ca^{++} is a sufficient stimulus to induce PNA binding in an increased proportion of keratinocytes propagated in the serum-free system.

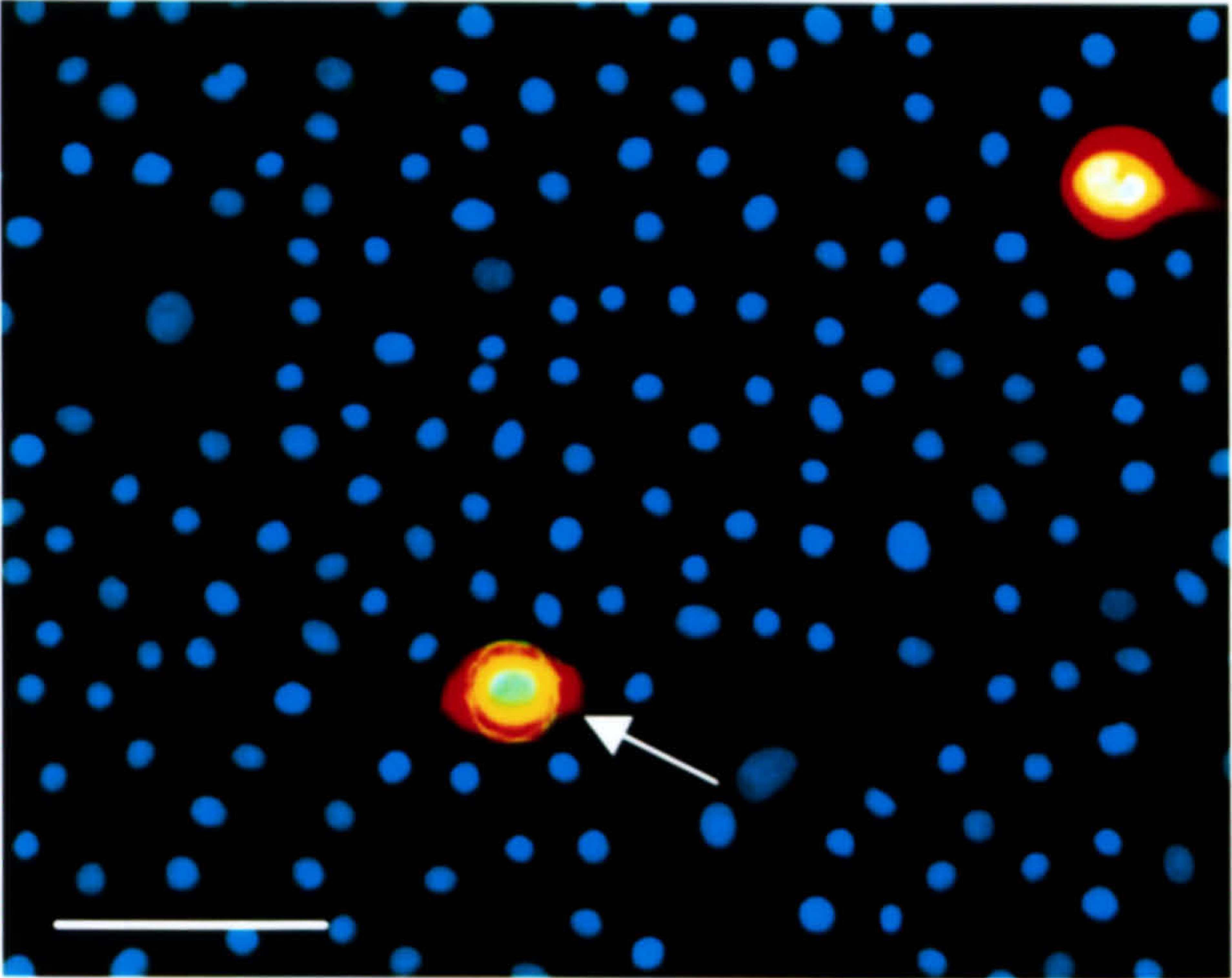
Figure 10

Figure 10
The induction of peanut agglutinin binding activity by high extracellular calcium

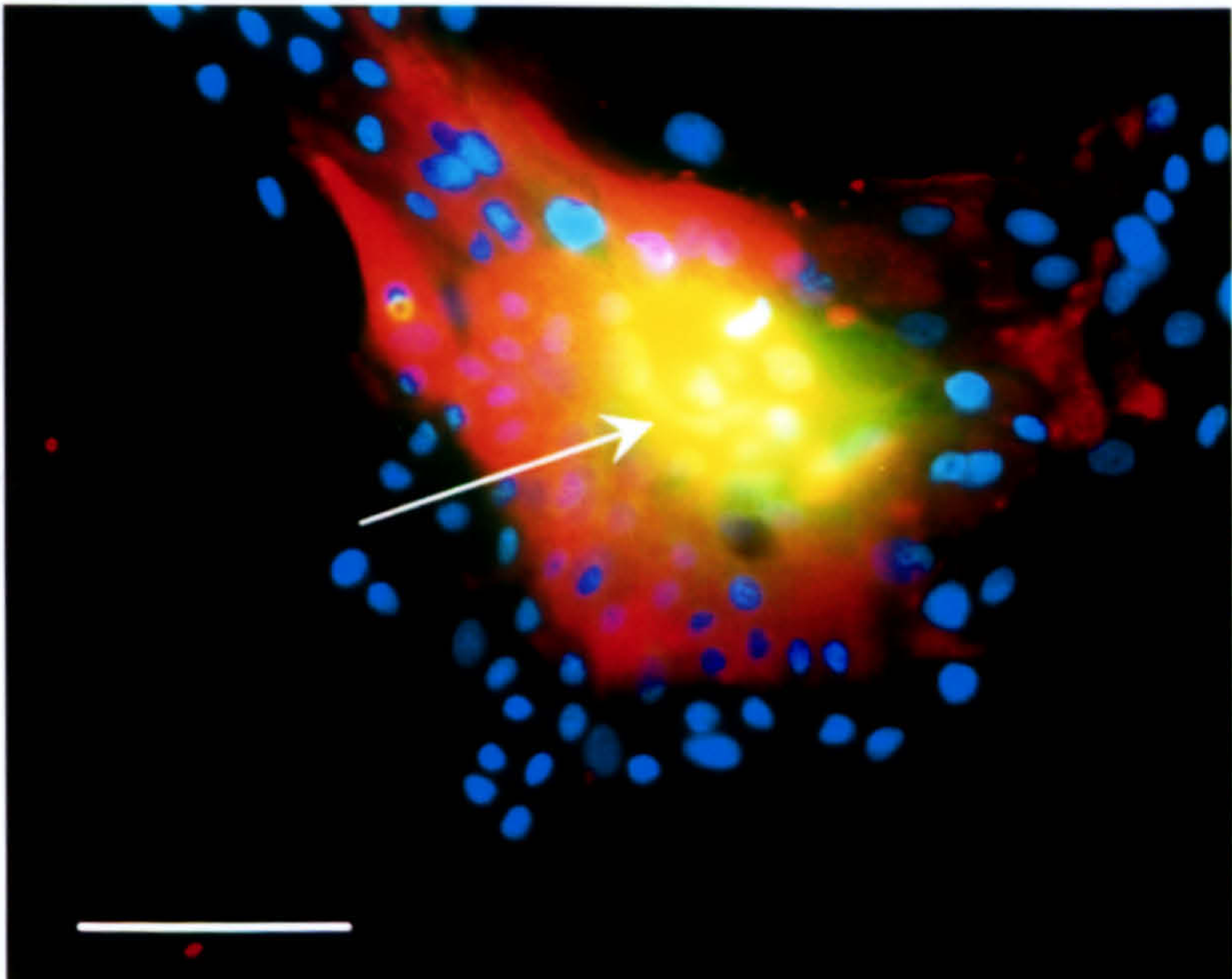
This figure shows two fluorescence micrographs of adherent keratinocytes maintained in 0.03mM extracellular Ca^{++} (*a*) or in 1mM extracellular Ca^{++} for 5 days (*b*). The cells have been stained for involucrin (red), peanut agglutinin binding (green) and the nuclei are stained blue. The antiserum used was anti-involucrin rabbit polyclonal , diluted 1:5. FITC-labelled peanut agglutinin was diluted 1:500 and DAPI was at a concentration of 0.1µg/ml (section 5.11). Scale bars represent 100µm.

The induction of peanut agglutinin binding activity by high extracellular calcium

a. low extracellular calcium



b. high extracellular calcium, 5 days



6.3 HIGH EXTRACELLULAR CALCIUM INCREASES CORNIFIED ENVELOPE COMPETENCE

6.3.1 Calcium induces envelope competence

Having demonstrated the induction of a group of differentiation-specific proteins by high extracellular Ca^{++}, the number of cells capable of the final phase of the differentiation process - cornified envelope (CE) assembly, was determined after growth in LKGM and HKGM. For envelope assembly to occur in a given cell, the presence of the envelope components and the enzymes to assemble them is required. In the serum-free culture system, keratinocytes grown in the presence of bovine pituitary extract (section 4.11.1) do not spontaneously assemble cornified envelopes (Boyce and Ham, 1983) in response to high extracellular Ca^{++} concentrations, but can be induced to do so using a non-physiological stimulus. Keratinocytes that have synthesised envelope components are artificially induced to assemble cornified envelopes by treatment with a Ca^{++}-specific ionophore (ionomycin or A23187) in the presence of high extracellular Ca^{++}, to allow the influx of extracellular Ca^{++} thereby activating TGk (Rice and Green, 1979). Keratinocytes incubated in high extracellular Ca^{++} for extended periods of time before treatment with ionophore have previously been shown to have an increased ability to assemble cornified envelopes compared with cells maintained in low extracellular Ca^{++} (Pillai *et al.,* 1990).

For this experiment, dishes of cells were seeded at different initial densities, calculated from proliferation assays (section 7.2.1), such that dishes harvested at different times after seeding would contain equivalent numbers of cells, thus minimising the effects of culture density on keratinocyte behaviour (section 8.3). These adherent keratinocytes were then grown in LKGM or HKGM for a period of up to 5 days before determining cell number and then incubating as a cell suspension in 10μM ionomycin for 4 hours. The detergent-insoluble cornified envelopes were extracted (section 5.10), visualised microscopically (Figure 11 *a*) and counted. Thus, the proportion of cells competent to assemble envelopes in response to ionophore under these conditions could be calculated (see Figure 11 *b*).

Growth in LKGM (green bars) for up to 5 days caused no significant change in the proportion of cells that were competent to assemble envelopes, demonstrating that the spontaneous rate of differentiation remained low under these conditions. Approximately 20% of keratinocytes grown in LKGM were competent to assemble

envelopes in response to ionomycin. Conversely, growth in HKGM (red bars) caused a progressive increase in the proportion of envelope-competent cells, rising to over 50% after 5 days, correlating with the increased involucrin protein levels induced under the same conditions. This indicates that high extracellular Ca^{++} induces sufficient cornified envelope components to allow cornified envelope assembly in an increased proportion of cells compared with cells maintained in LKGM.

6.3.2 Involucrin is a component of ionophore-induced cornified envelopes

In order to further validate involucrin as an indicator of cornified envelope competence and therefore differentiation, it was necessary to confirm that involucrin is a component of ionophore-induced cornified envelopes in this system. Keratinocytes grown in HKGM for 5 days were treated with ionomycin as described above to induce envelope formation (control cells were treated with DMSO) and the cells were then washed and lysed in CSK lysis buffer (section 4.11.2). These lysates were electrophoresed and immunoblotted for involucrin without prior clarification by centrifugation (Figure 12).

In the proteins extracted from control cells treated with DMSO, a single species reacted with anti-involucrin antiserum, migrating with an apparent molecular weight of 120kD, representing monomeric involucrin. However, in the proteins extracted from ionophore-treated cells, an additional species reacting with anti-involucrin antiserum was present, with an apparent molecular weight considerably exceeding 220kD. Furthermore, the development of this second species appeared to be at the expense of the faster migrating species. The slower migrating species most likely represents a subunit of the cornified envelope comprising involucrin, either complexed with itself or with other envelope components as a result of TGk activity (section 1.2.1B), and was resistant to boiling in SDS, suggesting that involucrin is covalently bound to the aggregate. Thus, involucrin is present in a high molecular weight protein aggregate in cells induced to form envelopes but not in control cells.

Figure 11

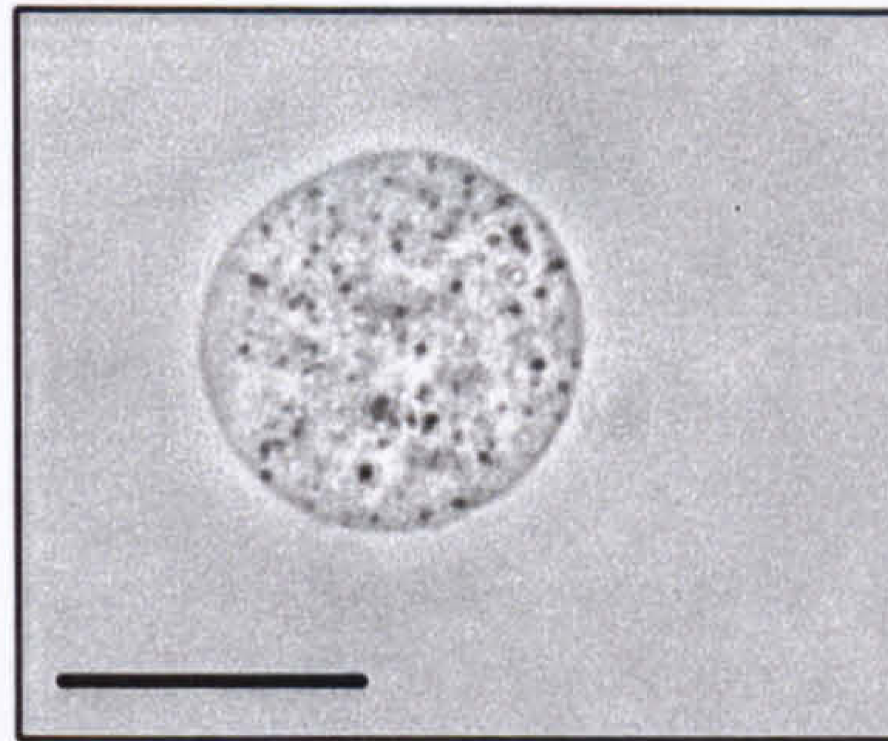
Figure 11
Calcium-induced competence to assemble cornified envelopes

a. Phase-contrast photomicrograph showing a single cornified envelope extracted from a keratinocyte following ionomycin treatment as described in section 5.10. Envelopes such as these were counted using a haemacytometer to determine the proportion of cells which were competent to assemble envelopes under in response to ionomycin. The scale bar represents a distance of 30μm.

b. Quantification of the proportion of keratinocytes competent to assemble cornified envelopes in response to ionomycin in the presence of 1mM extracellular Ca^{++}. The cells depicted by the green bars were maintained in 0.03mM Ca^{++} for up to 5 days before ionophore treatment. The cells depicted by the red bars were maintained in 0.03mM Ca^{++} and then shifted to 1mM extracellular Ca^{++} for various times of up to 5 days before treating with ionophore. Treatment with the vehicle for ionomycin (DMSO) did not induce envelope formation (not shown).

Calcium-induced competence to assemble cornified envelopes

a. a cornified envelope



b. the proportion of cells competent to assemble envelopes in response to ionophore

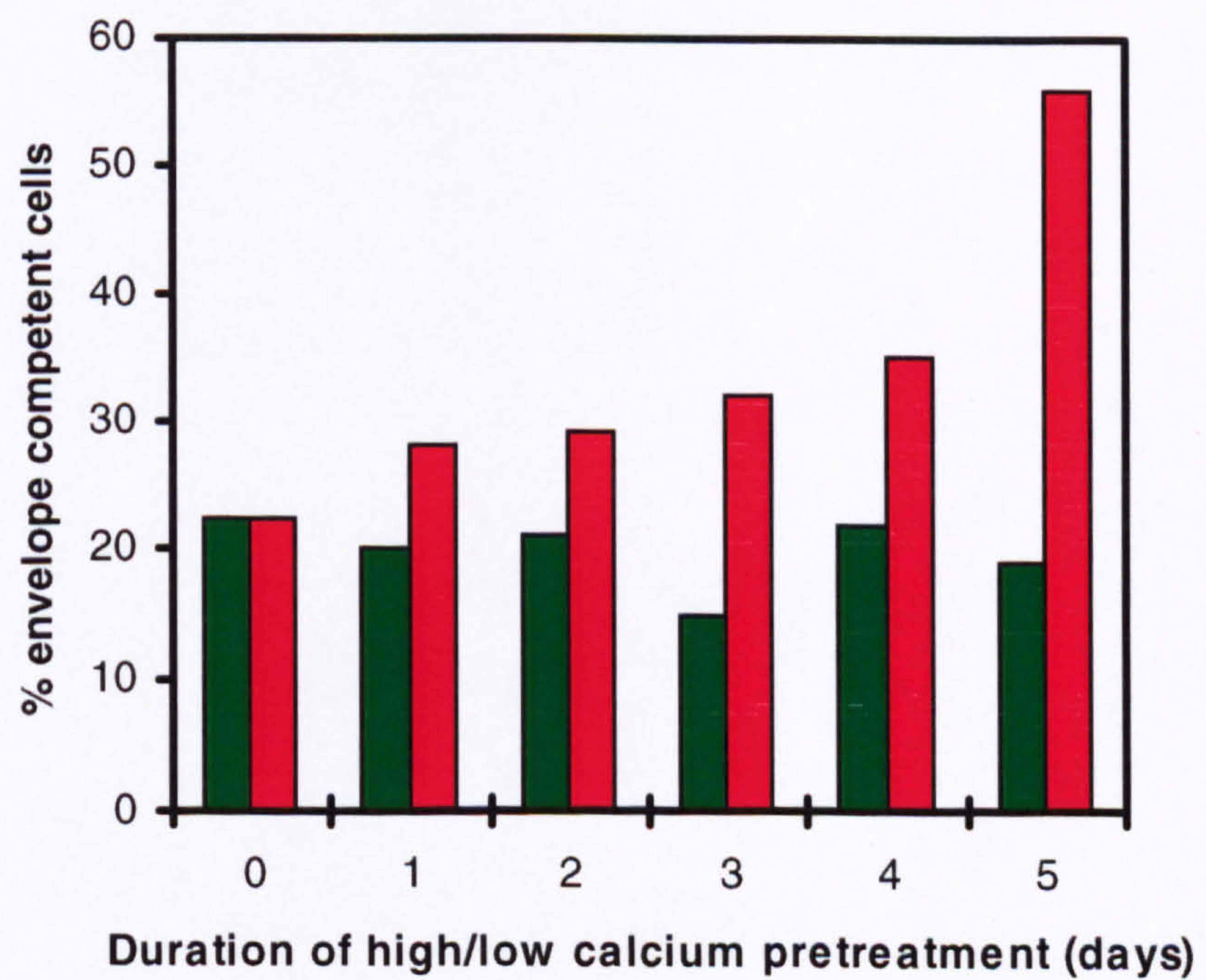
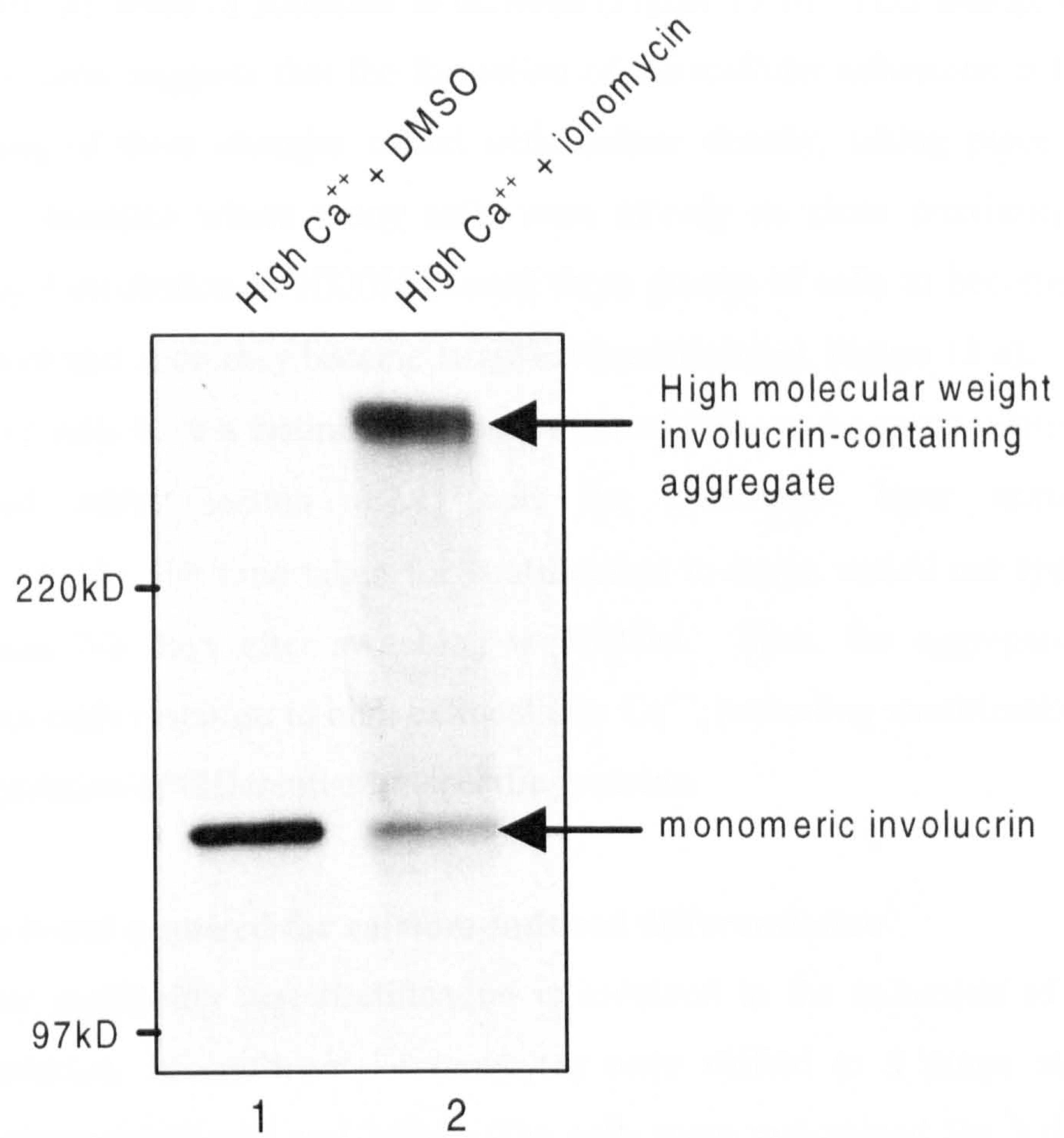


Figure 12

Figure 12
Involucrin is a component of ionomycin-induced cornified envelopes

Involucrin immunoblot prepared using proteins extracted from ionomycin-treated and control cells. These lysates were **not** clarified by centrifugation prior to electrophoresis (section 5.4). Molecular weight markers are shown. The antiserum used was anti-involucrin rabbit polyclonal (a generous gift from F. Watt, Imperial Cancer Research Fund, London), diluted 1:500.

Involucrin is a component of ionomycin-induced cornified envelopes



6.4 Calcium induces changes in keratinocyte distribution

A. The effect of 1mM extracellular calcium on keratinocyte distribution

In low Ca^{++} serum-free medium, subconfluent keratinocytes grew as a non-contiguous monolayer with very few obvious cell-cell contacts visible by phase-contrast light microscopy (Figure 13 *a*). However, switching to HKGM dramatically altered the keratinocyte distribution. The first change that was seen by light microscopy was the gradual coalescing of individual keratinocytes into larger coherent groups, a process that leaves few cells on the areas of substrate in between (Figure 13 *b*). This change in the distribution of the cells suggests that the formation of intercellular adhesions is taking place. The timing of these changes varied with culture density, taking place more rapidly at higher densities where many cells were already in close proximity (not shown). Continued incubation in HKGM caused these groups of cells to become ever more tightly packed and soon they became stratified (multilayered, Figure 13 *c*). These stratified groups of cells have a distinctive organisation with the upper strata comprising the differentiated cells (section 6.2.1) and the lowermost layer remaining undifferentiated. Again, the time taken for stratification to begin varied but typically stratification began 2-3 days after switching to HKGM. Thus, the aggregation of keratinocytes is an early response to high extracellular Ca^{++}, preceding stratification and the increased expression of differentiation-specific proteins.

B. Stratification is not required for calcium-induced differentiation

To investigate the possibility that stratification is involved in the induction of Ca^{++}-induced differentiation, subconfluent keratinocytes were shifted to a range of Ca^{++} concentrations between 0.03mM and 1mM. The cells were maintained for 5 days in these extracellular Ca^{++} concentrations before viewing and extracting proteins to immunoblot for involucrin expression (Figures 14 and 15 respectively).

In normal HKGM (1mM Ca^{++}), stratification always follows aggregation, but this was not the case at intermediate extracellular Ca^{++} concentrations, at least over the duration of these experiments. When cells were shifted from 0.03mM to extracellular Ca^{++} concentrations between 0.3mM and 0.6mM, the initial cell aggregation was indistinguishable from that seen in response to 1mM extracellular Ca^{++}. However, at

Figure 13

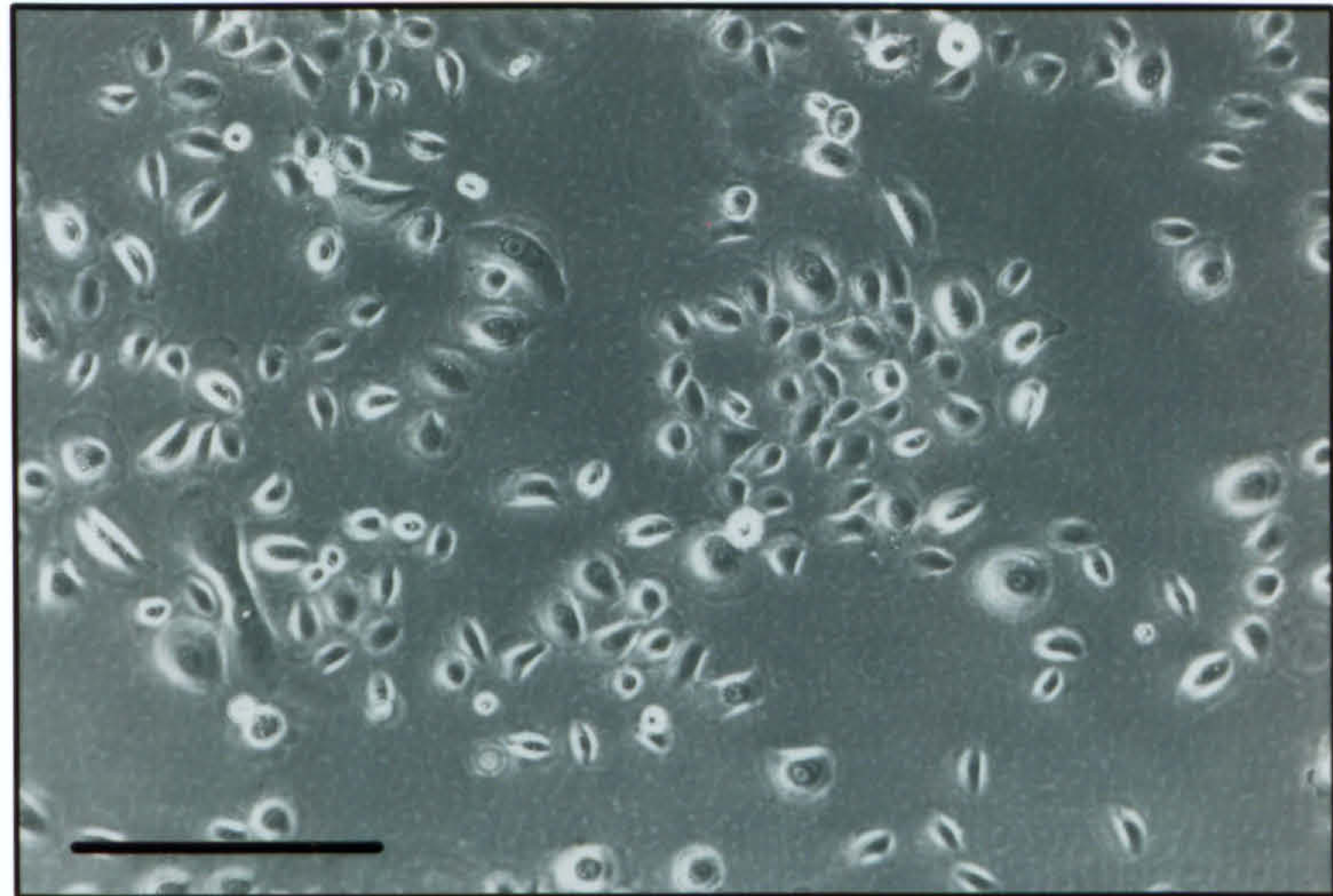
Figure 13

High extracellular calcium alters the distribution of adherent keratinocytes

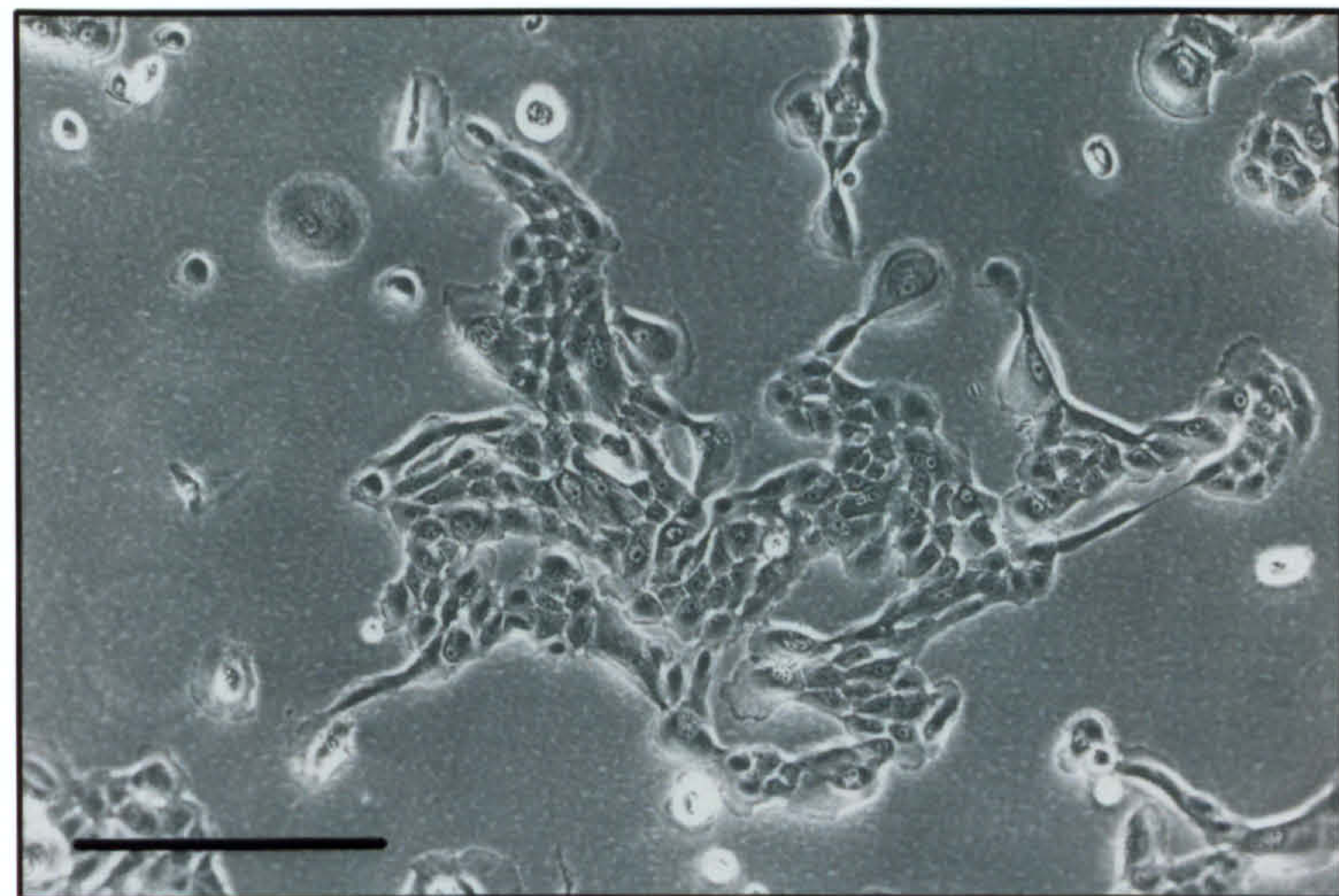
Phase-contrast photomicrographs of adherent subconfluent keratinocytes growing in 0.03mM Ca^{++} (*a*) and at 1 and 7 days after shifting to 1mM extracellular Ca^{++} (*b* and *c* respectively). The scale bars represent 200μm.

High extracellular calcium alters the distribution of adherent keratinocytes

a. low extracellular calcium



b. high extracellular calcium, 1 day



c. high extracellular calcium, 7 days

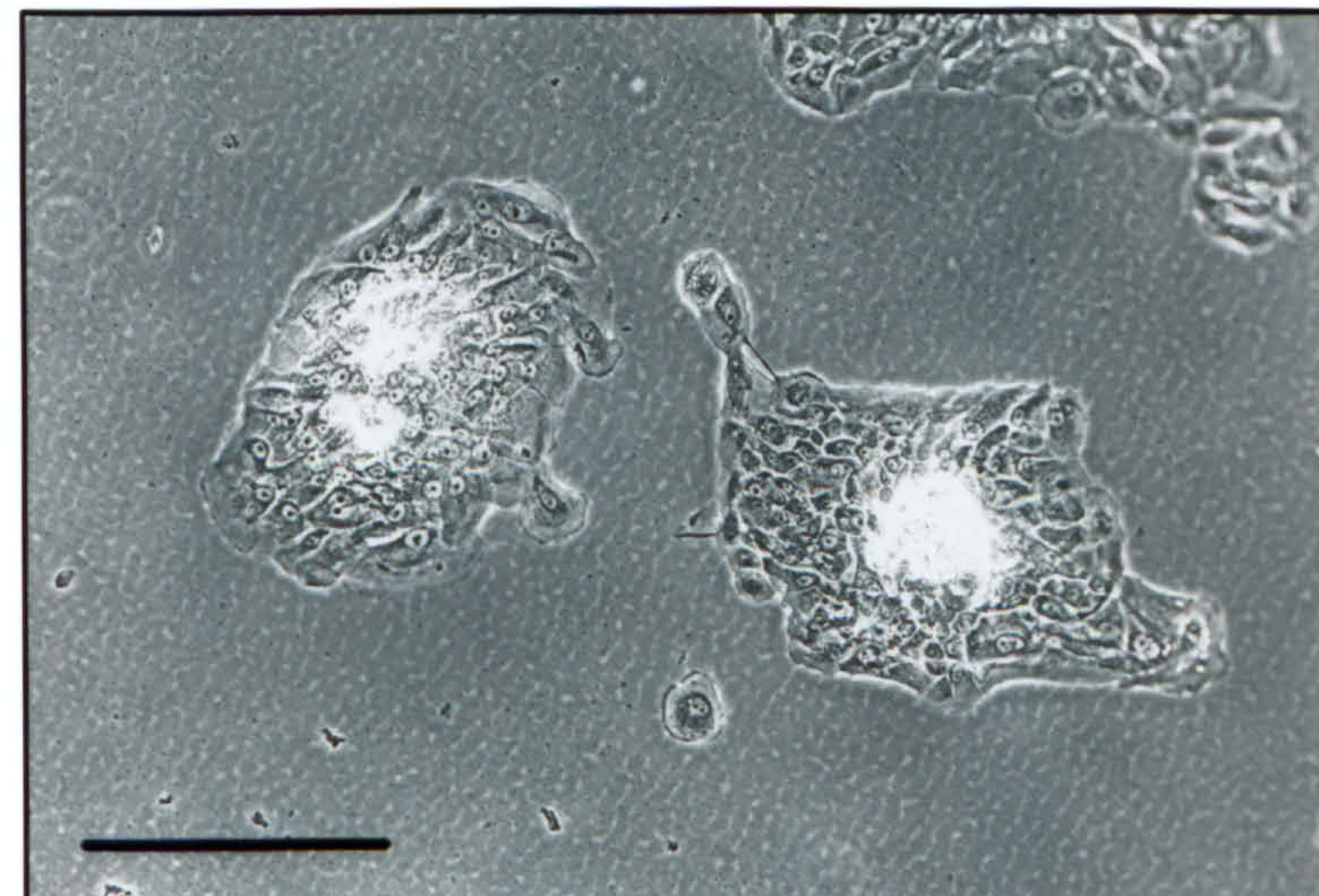


Figure 14

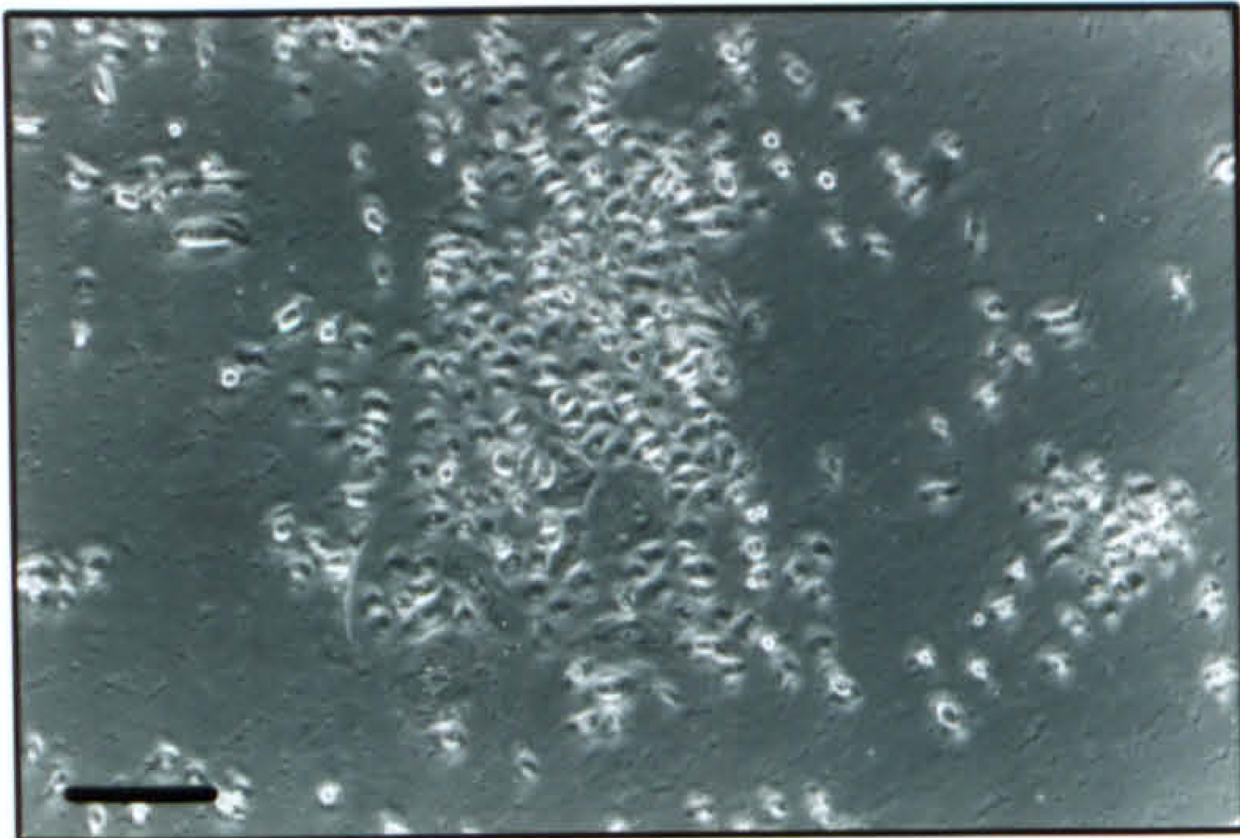
Figure 14

The effects of a range of calcium concentrations on keratinocyte distribution

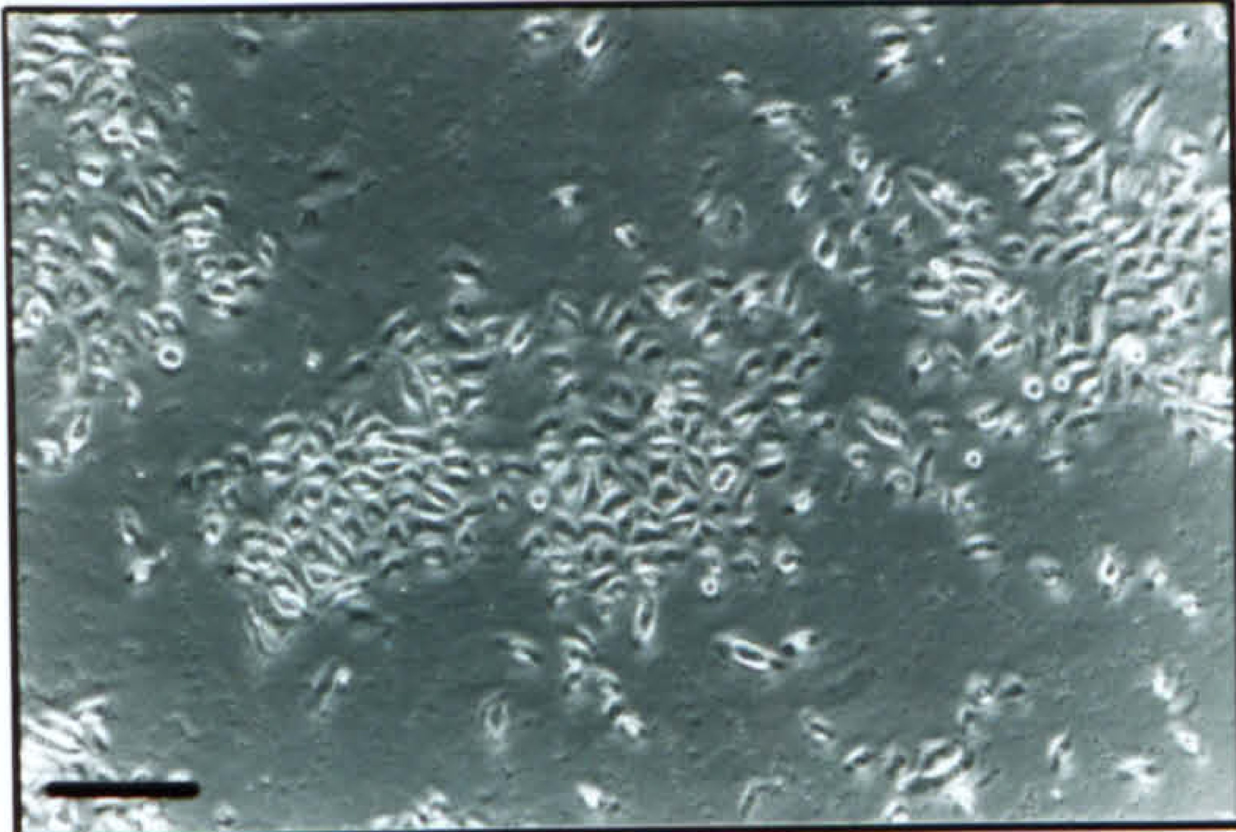
Phase-contrast photomicrographs of subconfluent adherent keratinocytes grown in extracellular Ca^{++} concentrations of 0.03, 0.1, 0.3, 0.45 and 2mM (*a*, *b*, *c*, *d* and *e*, respectively) for a duration of 5 days. Scale bars represent 200μm.

The effects of a range of calcium concentrations on keratinocyte distribution

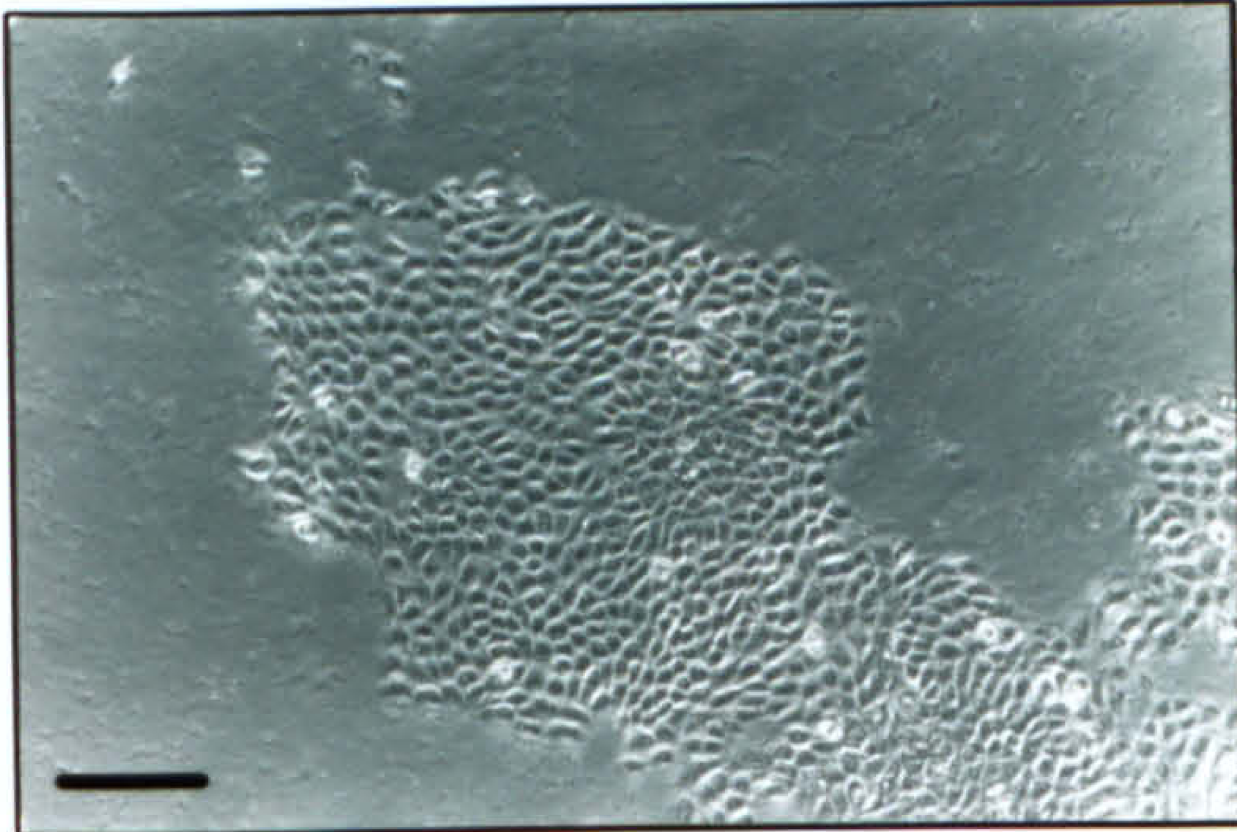
a. 0.03mM



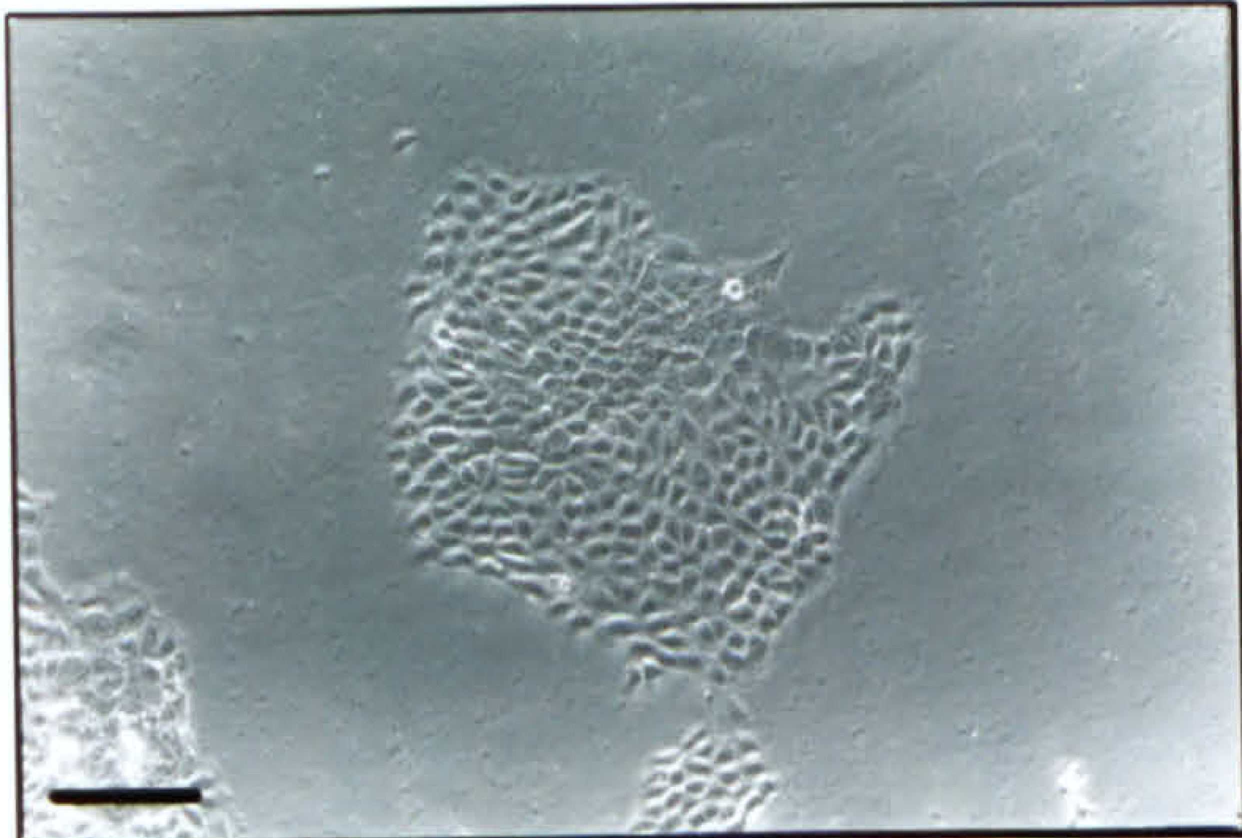
b. 0.1mM



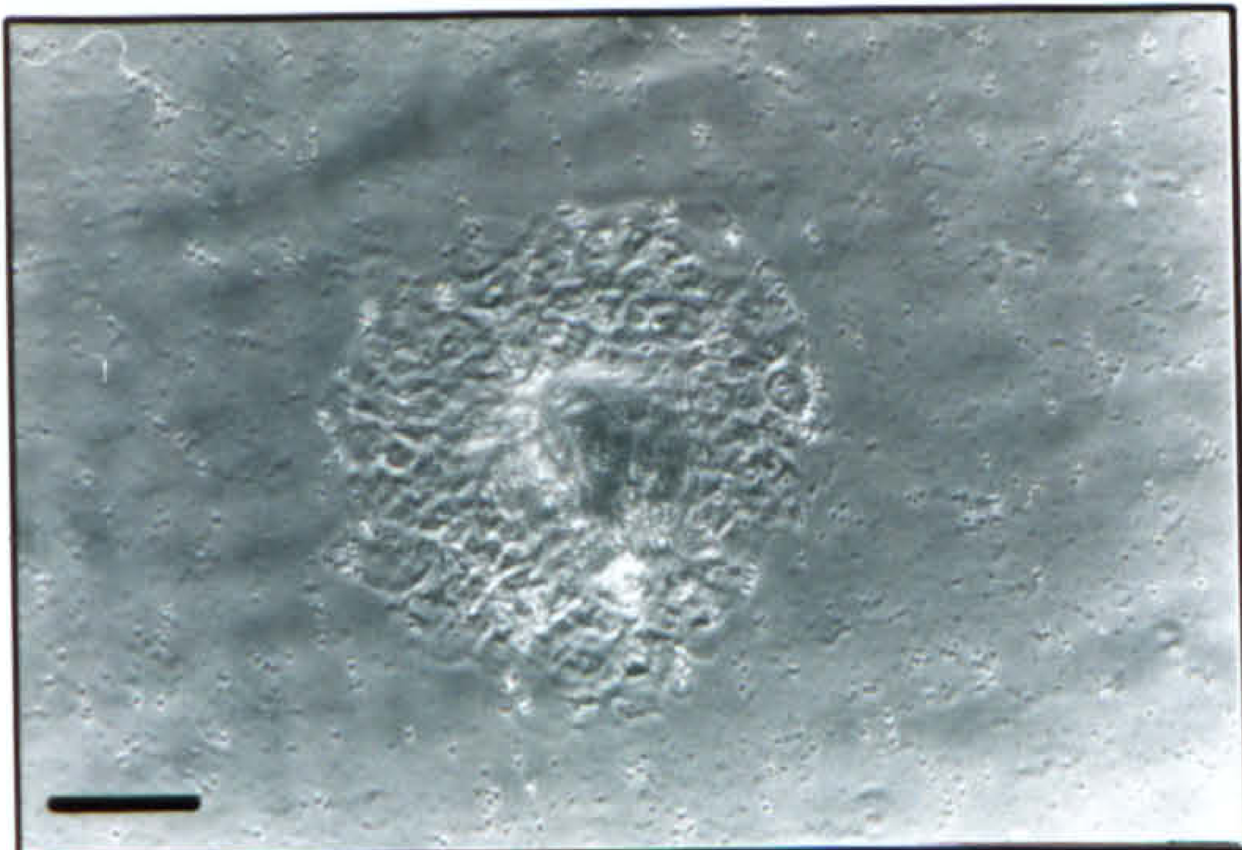
c. 0.3mM



d. 0.45mM



e. 2mM



these moderate Ca^{++} concentrations, no stratification took place. At Ca^{++} concentrations above 0.6mM, stratification occurred, suggesting that a higher Ca^{++} concentration is required for stratification than for the formation of monolayer aggregates of cells. Thus, at Ca^{++} concentrations between 0.03mM and 0.6mM, aggregation is induced, but not stratification.

At the two lowest extracellular Ca^{++} concentrations, the levels of involucrin expressed by the cells were very low, but at all concentrations of 0.3mM or greater, substantial involucrin expression occurred (Figure 15). There does not appear to be a fixed relationship between Ca^{++} concentration and the amount of involucrin expressed once the Ca^{++} concentration exceeds 0.45mM. Thus, a Ca^{++} concentration exceeding 0.45mM is sufficient to fully induce involucrin expression and further increases in Ca^{++} concentration have no further effect on involucrin expression. Taken together, the data from this experiment indicate that stratification is not a pre-requisite for differentiation, since intermediate Ca^{++} concentrations fully induce involucrin expression without stratification taking place. This conclusion is in agreement with Watt and Green, 1982.

6.5 CALCIUM INDUCED CELL-CELL ADHESIONS

To further characterise the changes in cell distribution that take place in response to switching from LKGM to HKGM, the subcellular localisation of a number of cell-adhesion proteins was examined by confocal immunofluorescence microscopy. Keratinocytes were grown on glass chamber slides in LKGM and then shifted to HKGM at suitable intervals such that the cells had been incubated in 1mM extracellular Ca^{++} for 0, 4 or 24 hours at the time of fixation. These times were chosen to coincide with the period when the greatest changes in cell distribution took place in response to Ca^{++}-shift.

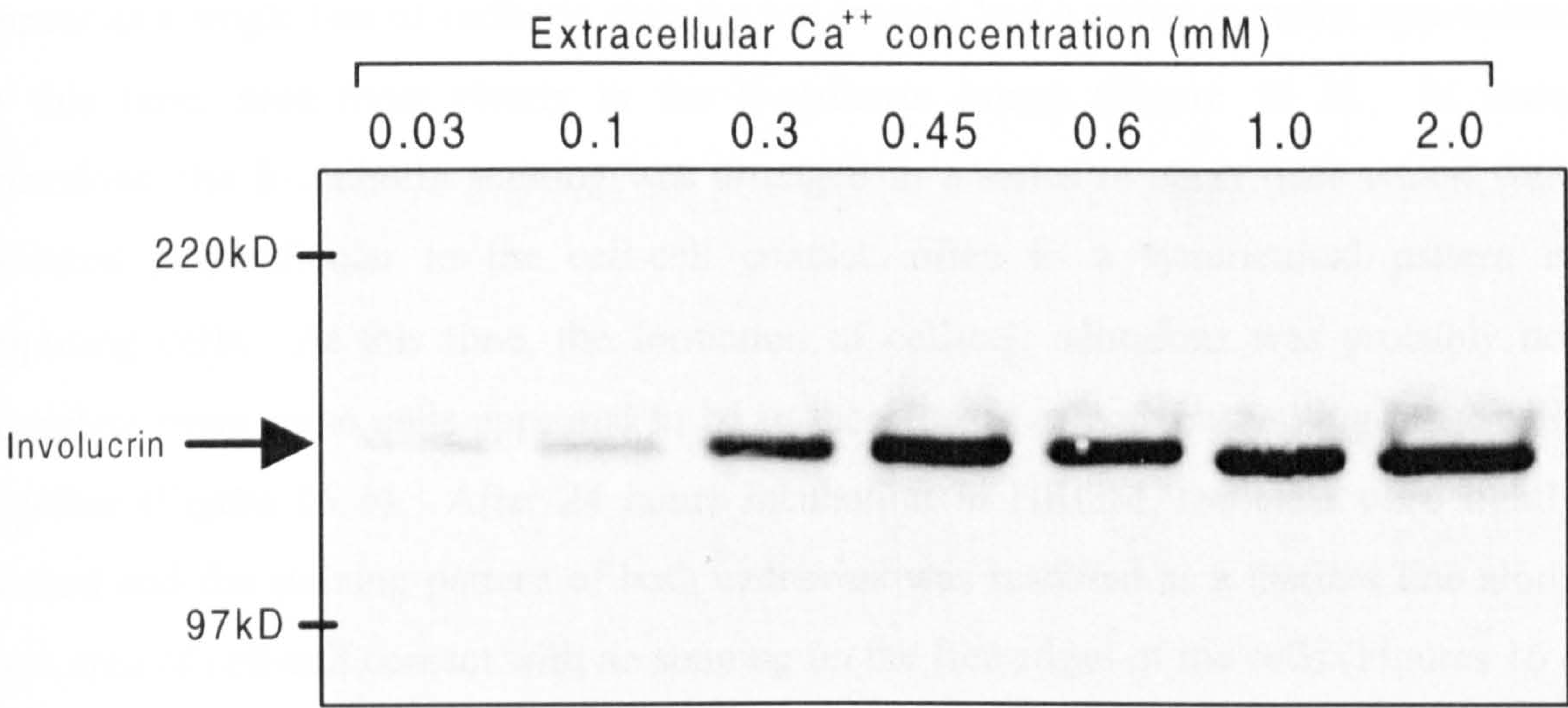
Figure 15

Figure 15

The induction of involucrin expression by a range of extracellular calcium concentrations

This immunoblot for involucrin shows the effects of a range of extracellular Ca^{++} concentrations on involucrin protein levels 5 days after switching subconfluent keratinocytes from 0.03mM Ca^{++} to the indicated Ca^{++} concentrations. This experiment was performed in collaboration with Dr. E.K. Parkinson, Beatson Institute. The antiserum used anti-involucrin rabbit polyclonal, diluted 1:500.

The induction of involucrin expression by a range of extracellular calcium concentrations



6.5.1 High extracellular calcium induces the formation of adherens junctions

A. Subcellular localisation of the classical cadherins

In subconfluent cultures maintained in LKGM, the staining for both E- and P-cadherin was largely diffuse although occasional intercellular staining for E-cadherin could be seen (Figure 16 *a*, arrow), indicating that 0.03mM Ca^{++} does not completely suppress the formation of adherens junctions. Much of the P-cadherin in cells grown under these conditions appeared to be localised to the cytoplasm in the vicinity of the nuclei (Figure 17 *a*). Four hours after switching to HKGM, both cadherins were present in areas of cell-cell contact, corresponding to the redistribution of the cells and the formation of adherens junctions (Figures 16 *b* and 17 *b*). These newly formed adhesions did not appear as a single line of cadherin staining but instead had a rather complex appearance at this time, seen most clearly in the E-cadherin image (Figure 16 *b*). In many adhesions, the E-cadherin staining was arranged in a series of short lines which were oriented perpendicular to the cell-cell contact, often in a symmetrical pattern in opposing cells. At this time, the formation of cell-cell adhesions was probably not complete since some cells appeared to be in the process of actively pulling other cells together (Figure 16 *b*). After 24 hours incubation in HKGM, the cells were tightly packed and the staining pattern of both cadherins was resolved as a distinct line along each area of cell-cell contact with no staining on the free edges of the cells (Figures 16 *c* and 17 *c*).

To further elucidate the nature of the cadherin translocation process, the importance of the actin cytoskeleton and the microtubule network was determined. The keratinocytes were treated with either cytochalasin D to disrupt the actin cytoskeleton, or with nocodazole to disrupt the intracellular microtubules (e.g., Tomasek and Hay, 1984). Cells were treated in this way for 30 minutes before switching from 0.03mM Ca^{++} to 1mM extracellular Ca^{++} for a duration of 4 hours and were then stained for both E- and P-cadherin. Nocodazole had no effect on the redistribution of E-cadherin or P-cadherin in response to high extracellular Ca^{++} (Figures 16 *e* and 17 *e*), despite causing the disruption of the microtubules (Figure 18 *b*) which were visible as discrete filamentous structures when stained using anti-α-tubulin antiserum before treatment (Figure 18 *a*). However, treating the cells with cytochalasin D suppressed cadherin translocation (Figures 16 *d*, and 17 *d*) and disrupted the actin filaments (Figure 19 *c*).

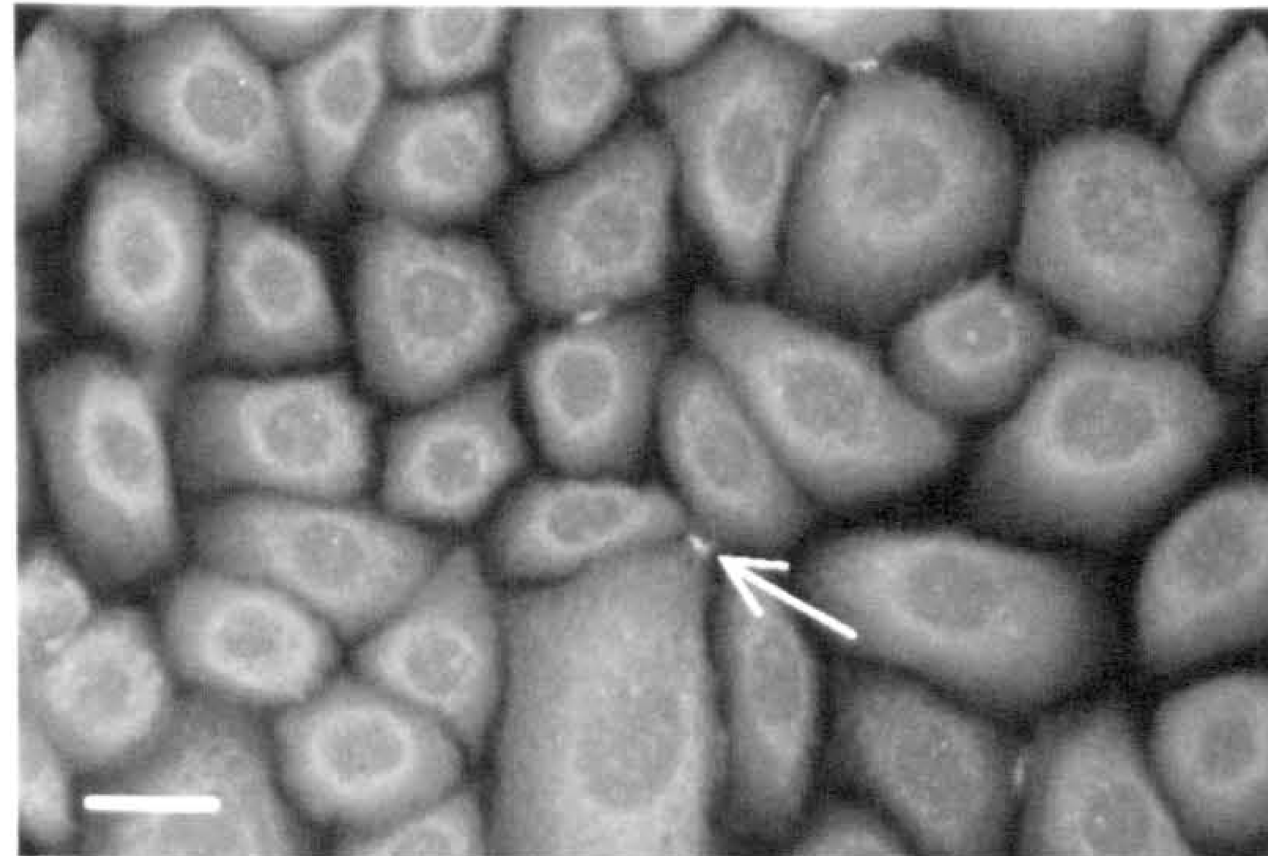
Figure 16

Figure 16
High extracellular calcium induces E-cadherin relocalisation

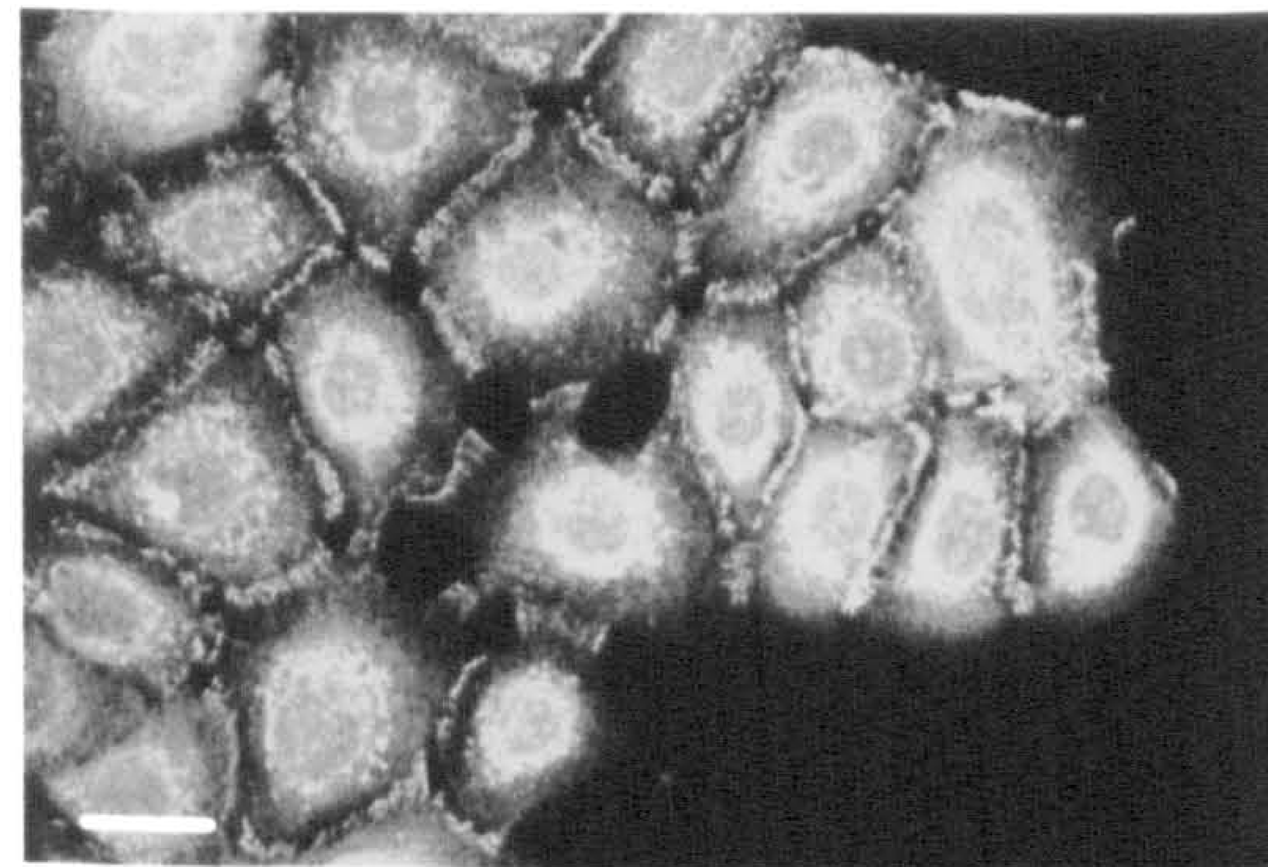
Confocal immunofluorescence micrographs of adherent keratinocytes stained for E-cadherin after growing in 0.03mM (*a*) and 4 and 24 hours after shifting to 1mM extracellular Ca^{++} (*b* and *c* respectively). Panel *d* shows cells pre-treated with 5μg/ml cytochalasin D for 30 minutes before shifting to 1mM Ca^{++} for 4 hours in the presence of the drug. Panel *e* shows cells pre-treated with 2.5μg/ml nocodazole for 30 minutes before switching to 1mM Ca^{++} for 4 hours in the presence of the drug. The antiserum used was DECMA-1 diluted 1:100, visualised with anti-rat-FITC diluted 1:125. The scale bars represent 25μm.

High extracellular calcium induces E-cadherin relocalisation

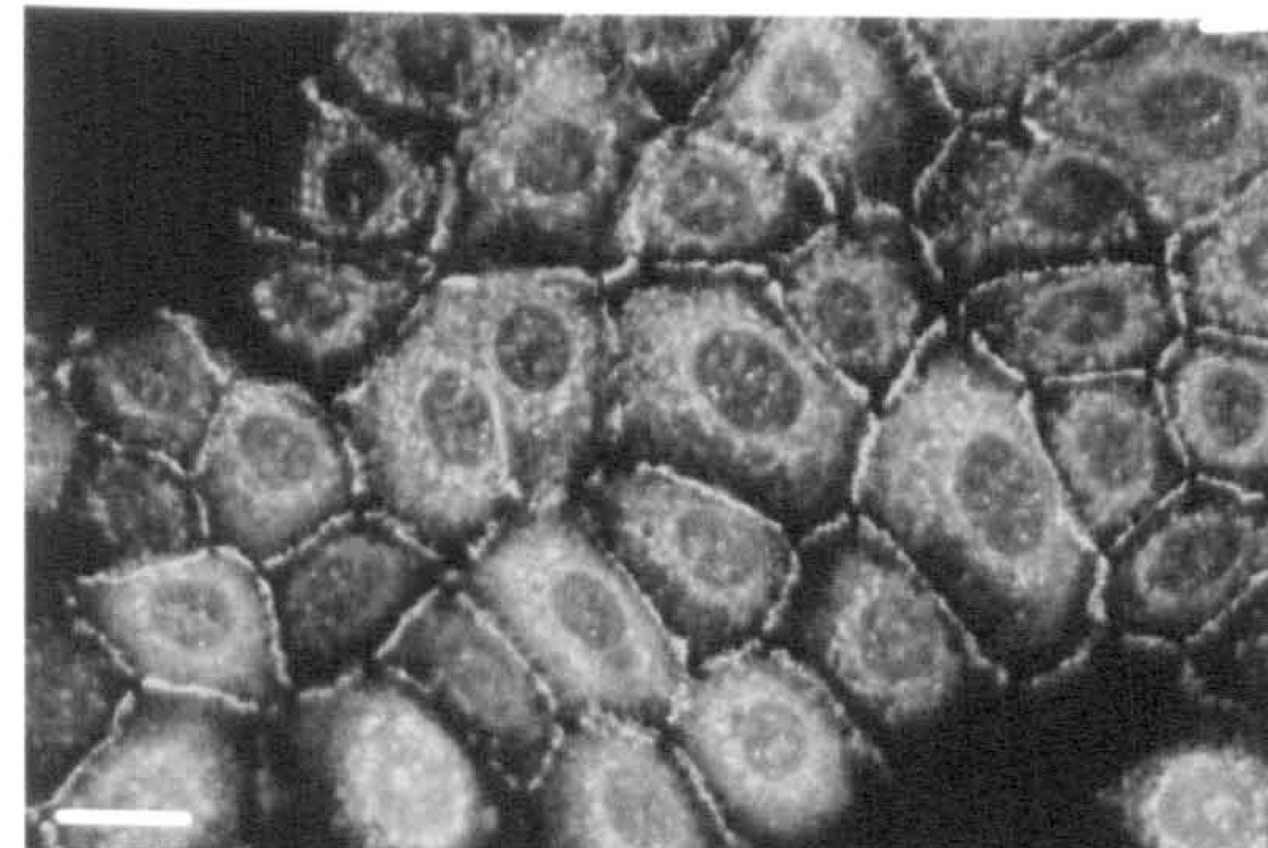
a. low extracellular calcium



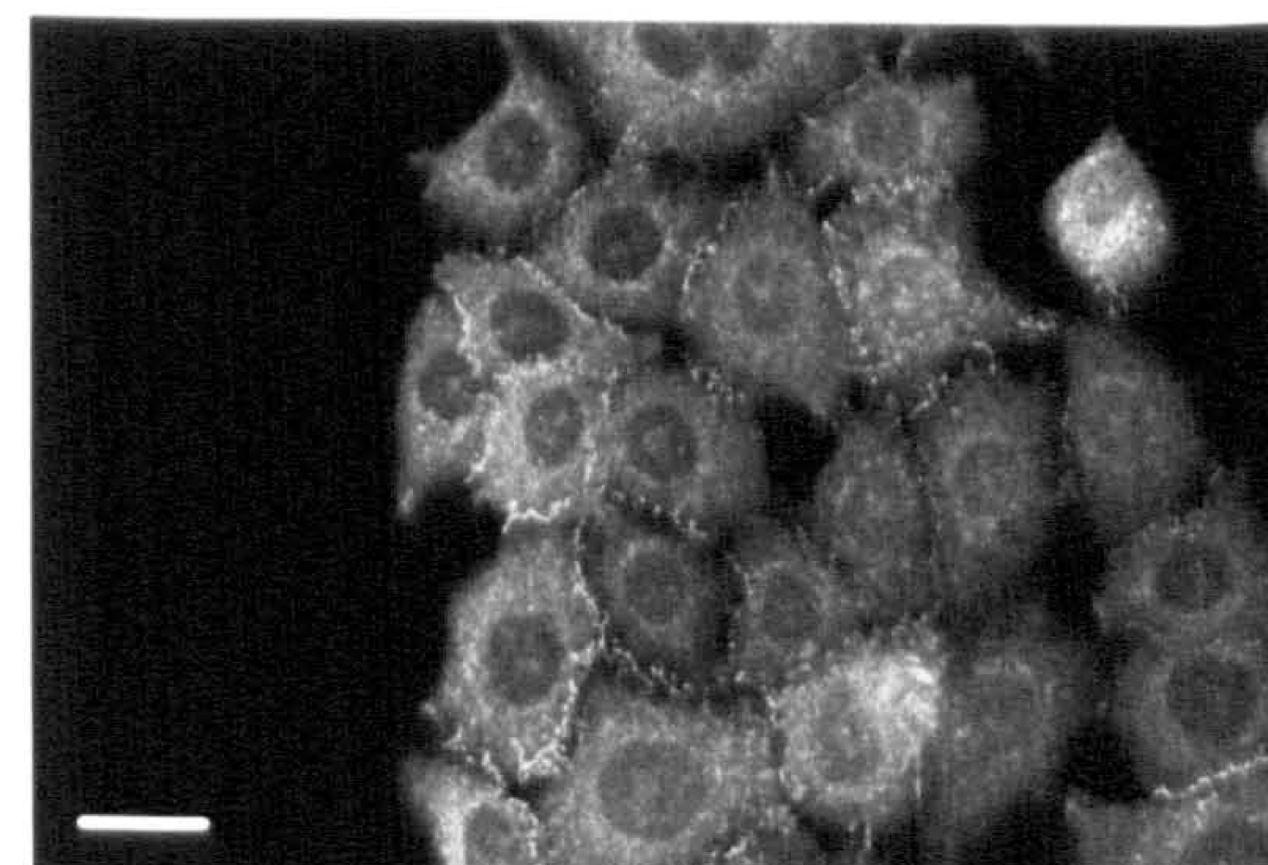
b. high extracellular calcium, 4 hours



c. high extracellular calcium, 24 hours



d. high extracellular calcium, 4 hours + cytochalasin D



e. high extracellular calcium, 4 hours + nocodazole

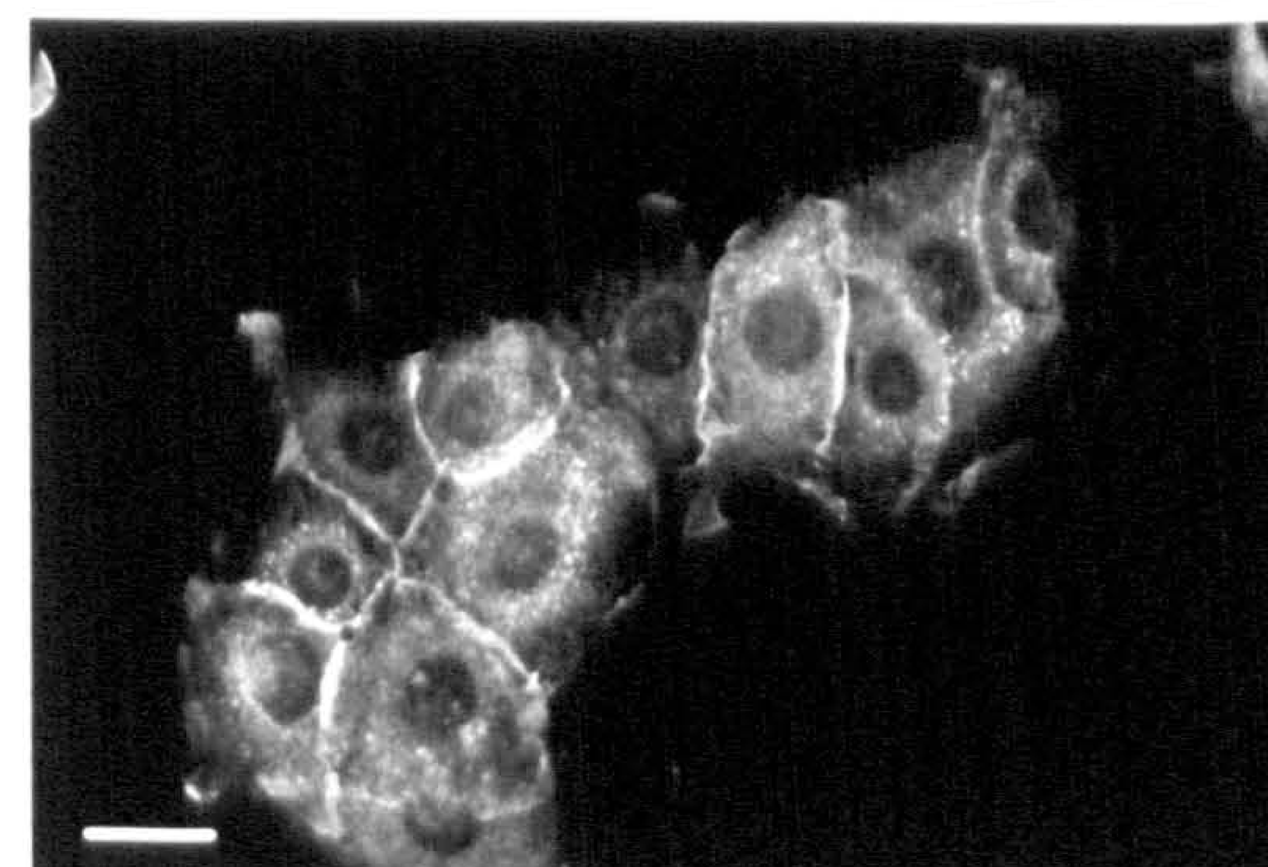


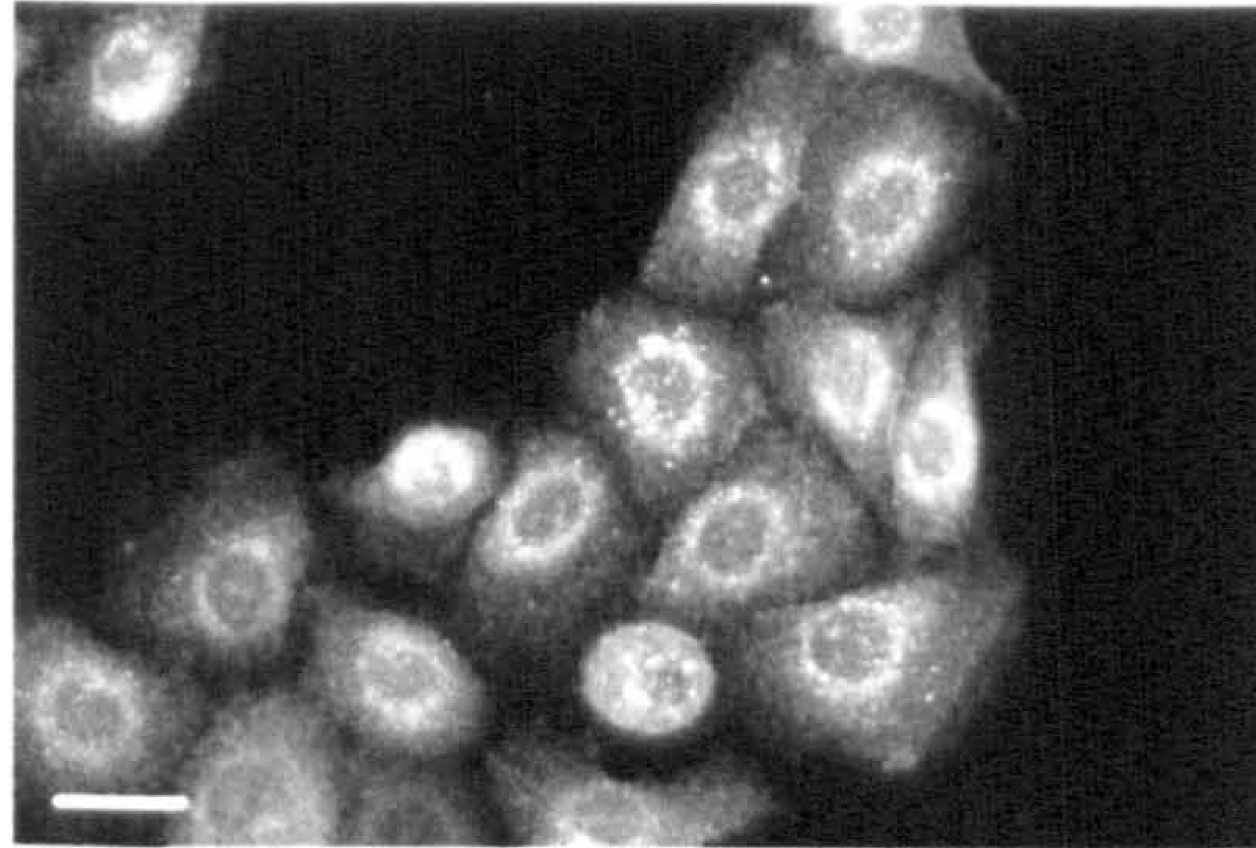
Figure 17

Figure 17
High extracellular calcium induces P-cadherin relocalisation

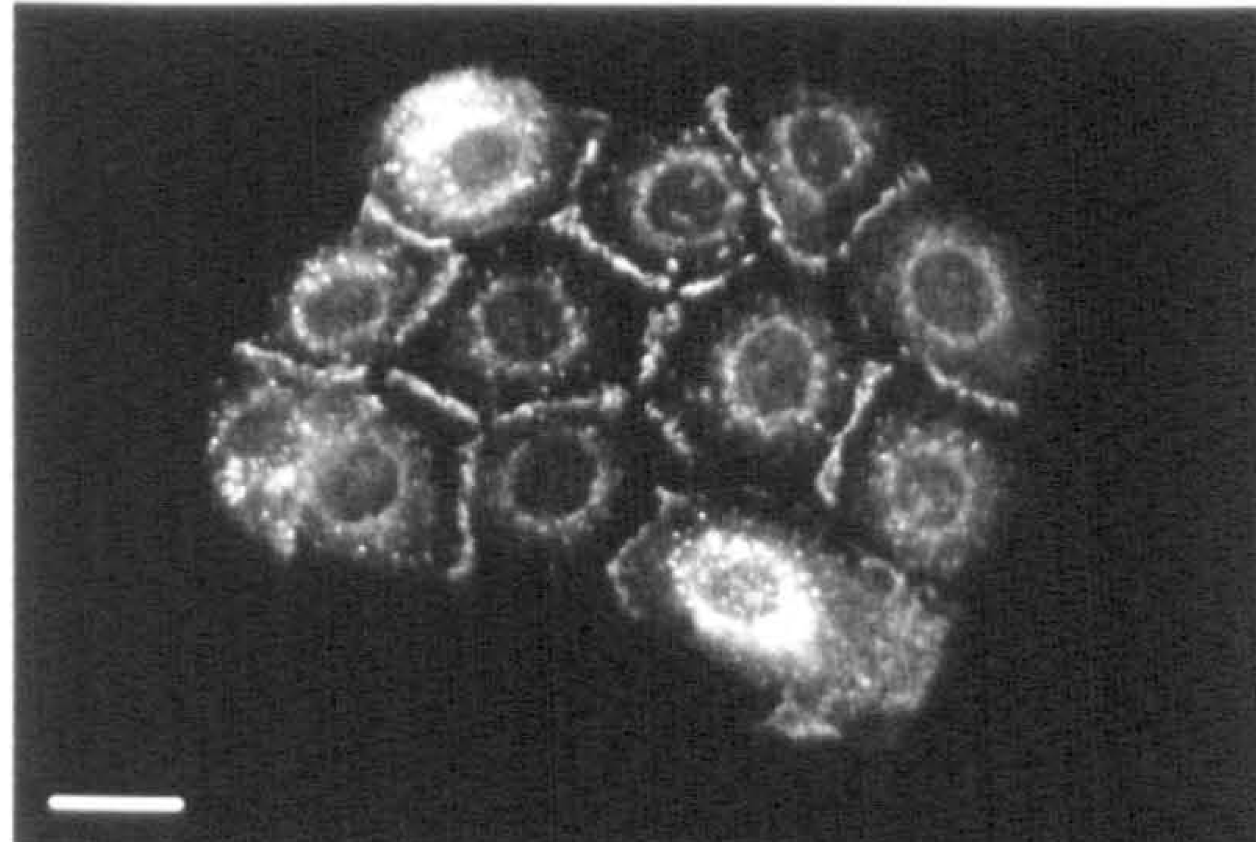
Confocal immunofluorescence micrographs of adherent keratinocytes stained for P-cadherin after growing in 0.03mM (*a*) and 4 and 24 hours after shifting to 1mM extracellular Ca^{++} (*b* and *c* respectively). Panel *d* shows cells pre-treated with 5μg/ml cytochalasin D for 30 minutes before shifting to 1mM Ca^{++} for 4 hours. Panel *e* shows cells pre-treated with 2.5μg/ml nocodazole for 30 minutes before switching to 1mM Ca^{++} for 4 hours in the presence of the drug. The antiserum used was mAb 6A9 (a gift from M. Wheelock, University of Toledo, Ohio) diluted 1:25, visualised with anti-mouse-FITC diluted 1:100. The scale bars represent 25μm.

High extracellular calcium induces P-cadherin relocalisation

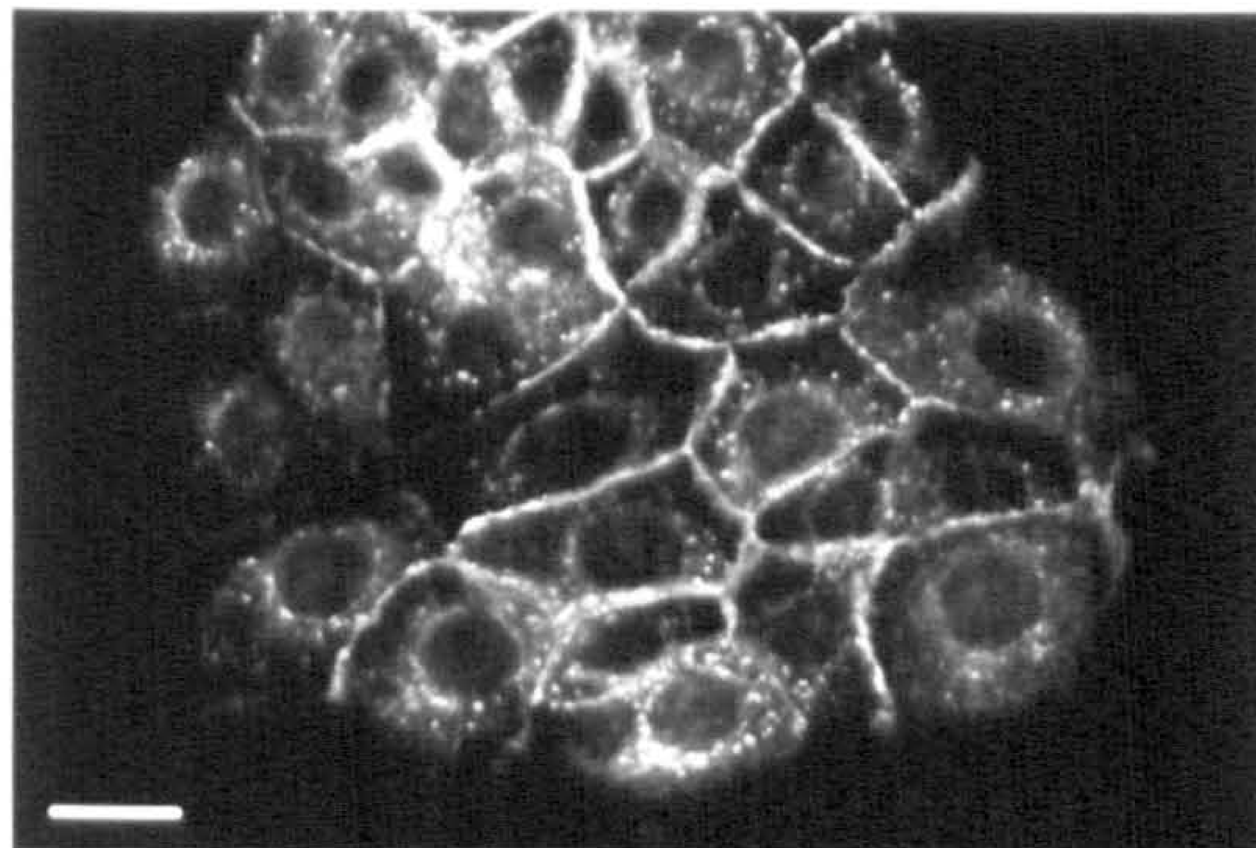
a. low extracellular calcium



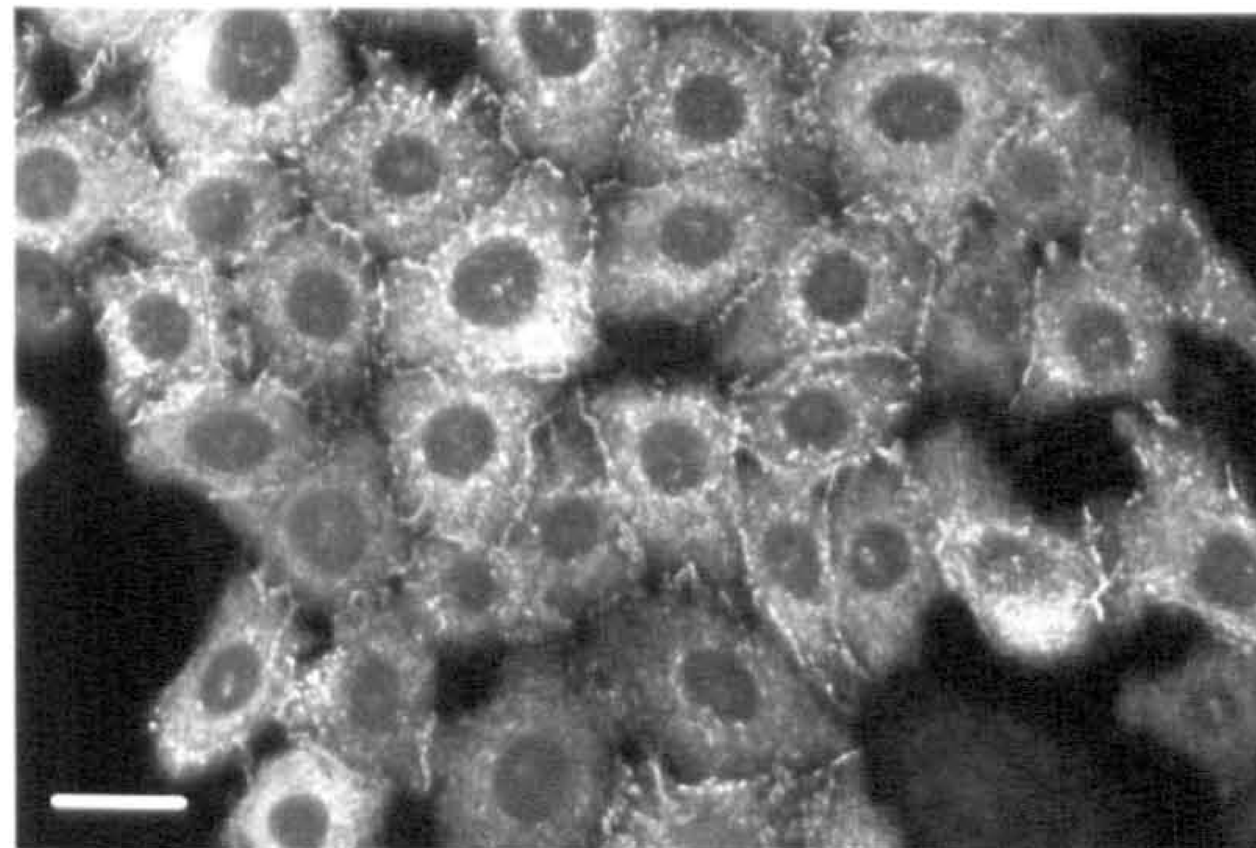
b. high extracellular calcium, 4 hours



c. high extracellular calcium, 24 hours



d. high extracellular calcium, 4 hours + cytochalasin D



e. high extracellular calcium, 4 hours + nocodazole

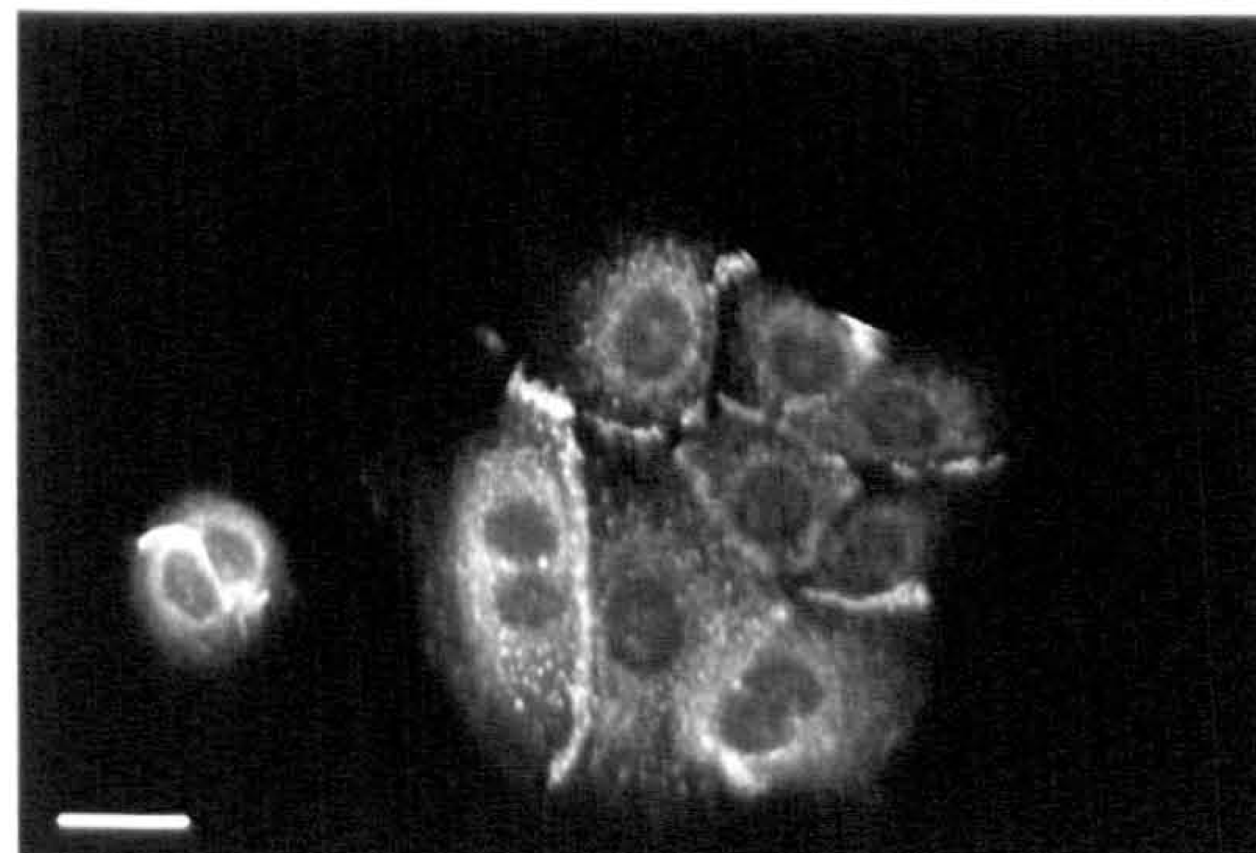


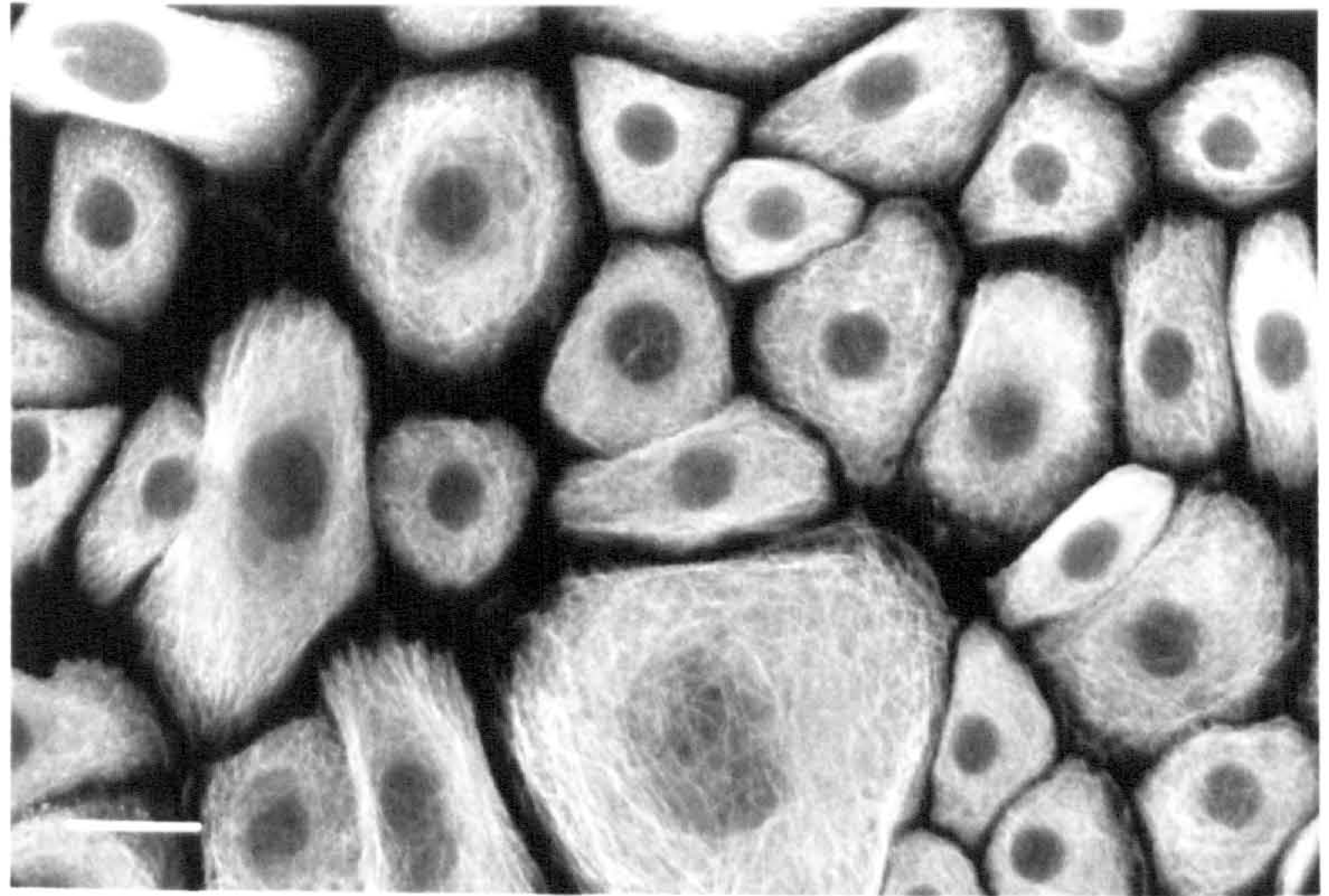
Figure 18

Figure 18
The effects of nocodazole and cytochalasin D on microtubule arrangement

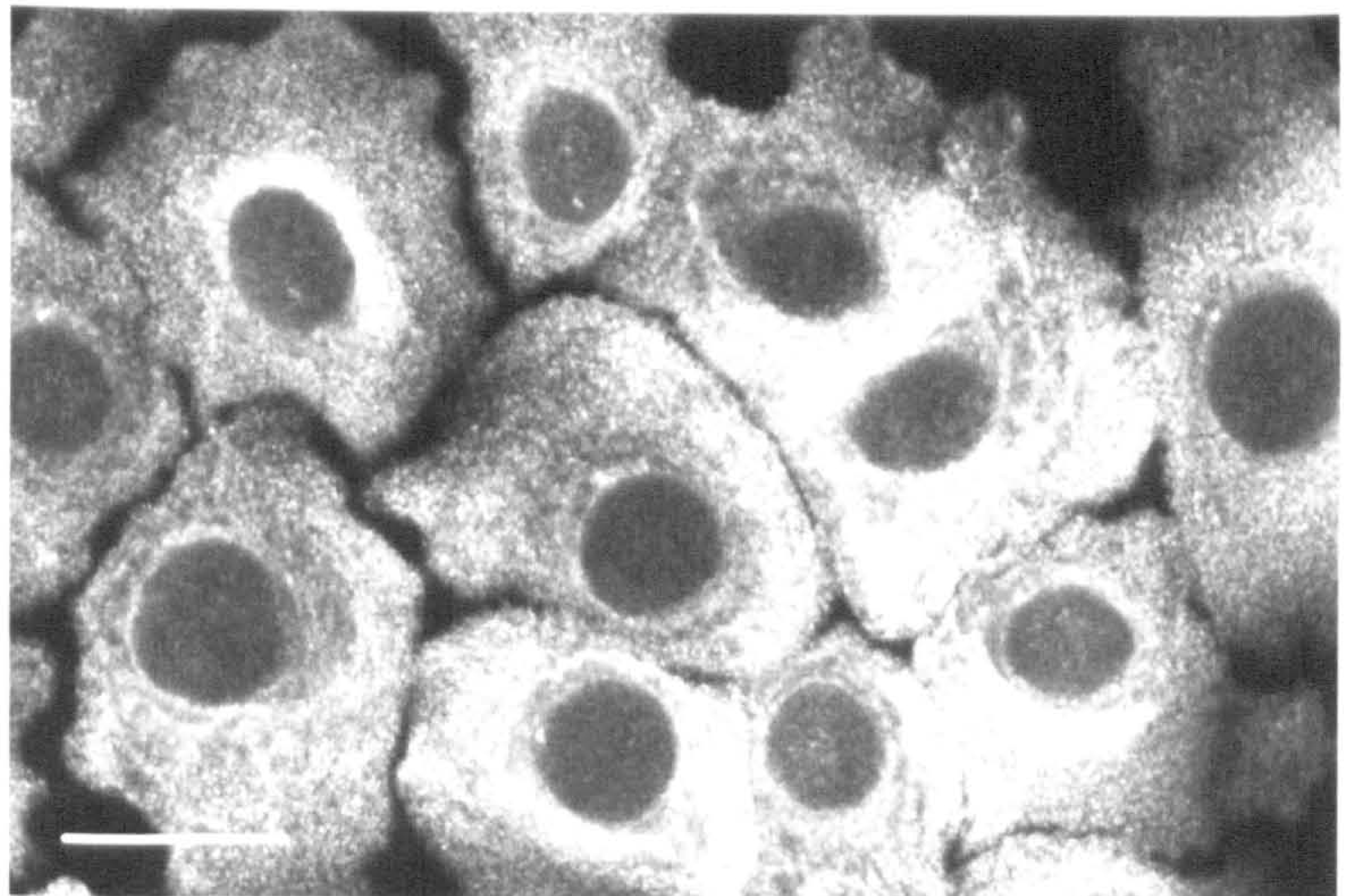
Confocal immunofluorescence images of adherent keratinocytes grown in 0.03mM Ca^{++} stained for α-tubulin before (*a*) and after treatment with 2.5μg/ml nocodazole for 4 hours (*b*), or 5μg/ml cytochalasin D for 4 hours (*c*). The antiserum used was anti-α-tubulin diluted 1:1000, visualised with anti-mouse-FITC diluted 1:100. Scale bars represent 25μm.

The effects of nocodazole and cytochalasin D on microtubule arrangement

a. low extracellular calcium, no drug



b. low extracellular calcium, nocodazole for 4 hours



c. low extracellular calcium, cytochalasin D for 4 hours

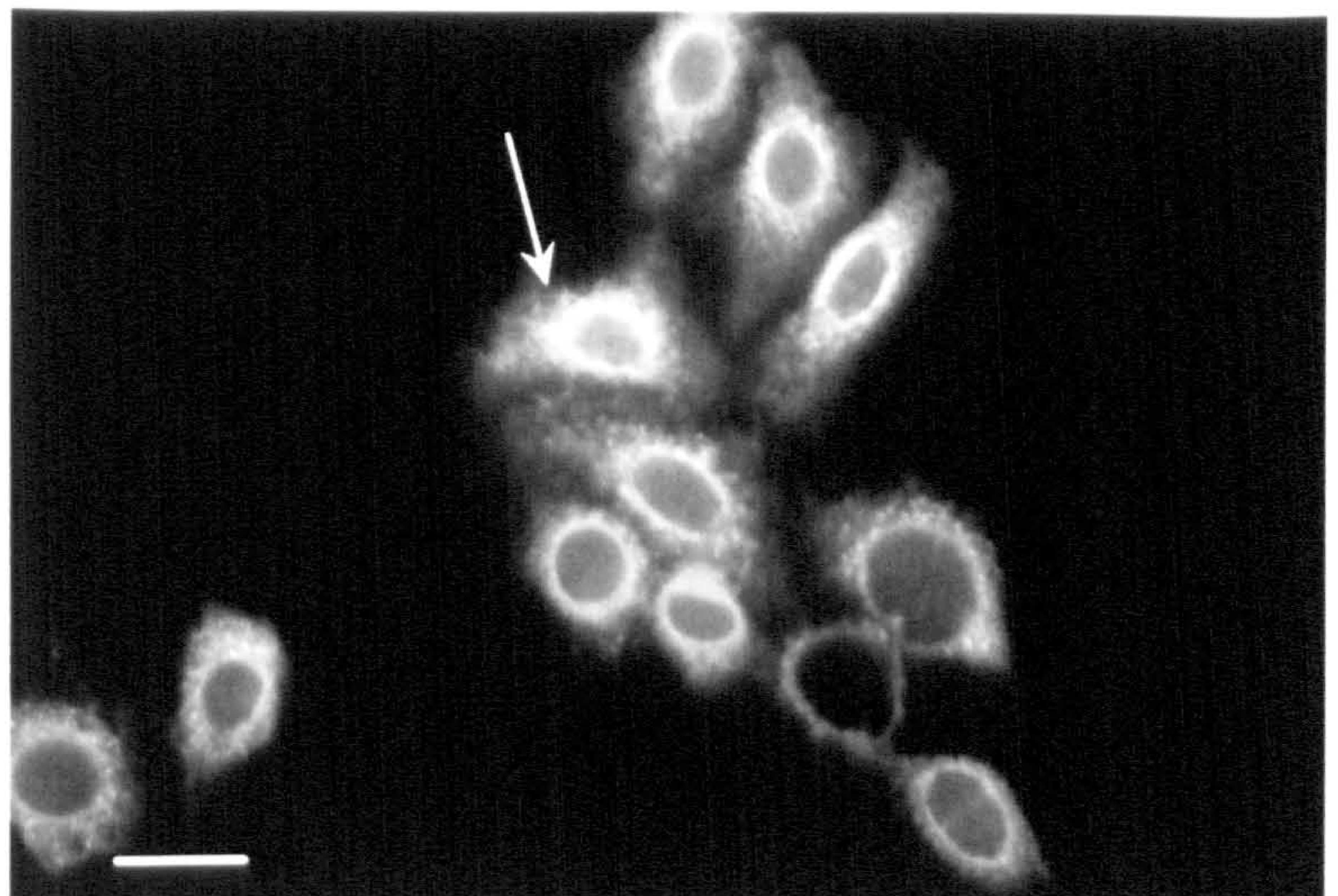


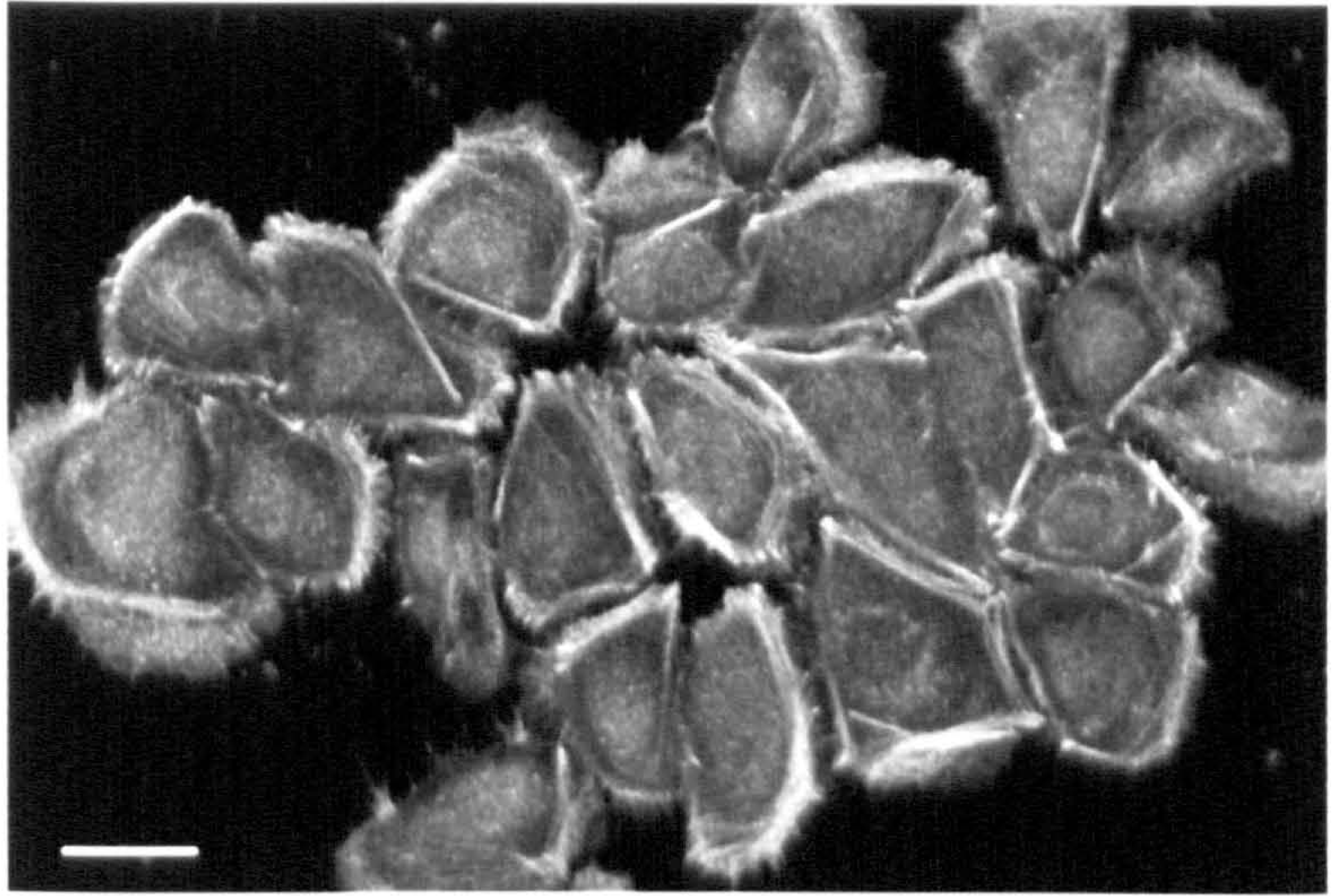
Figure 19

Figure 19
Cytochalasin D-induced actin disruption

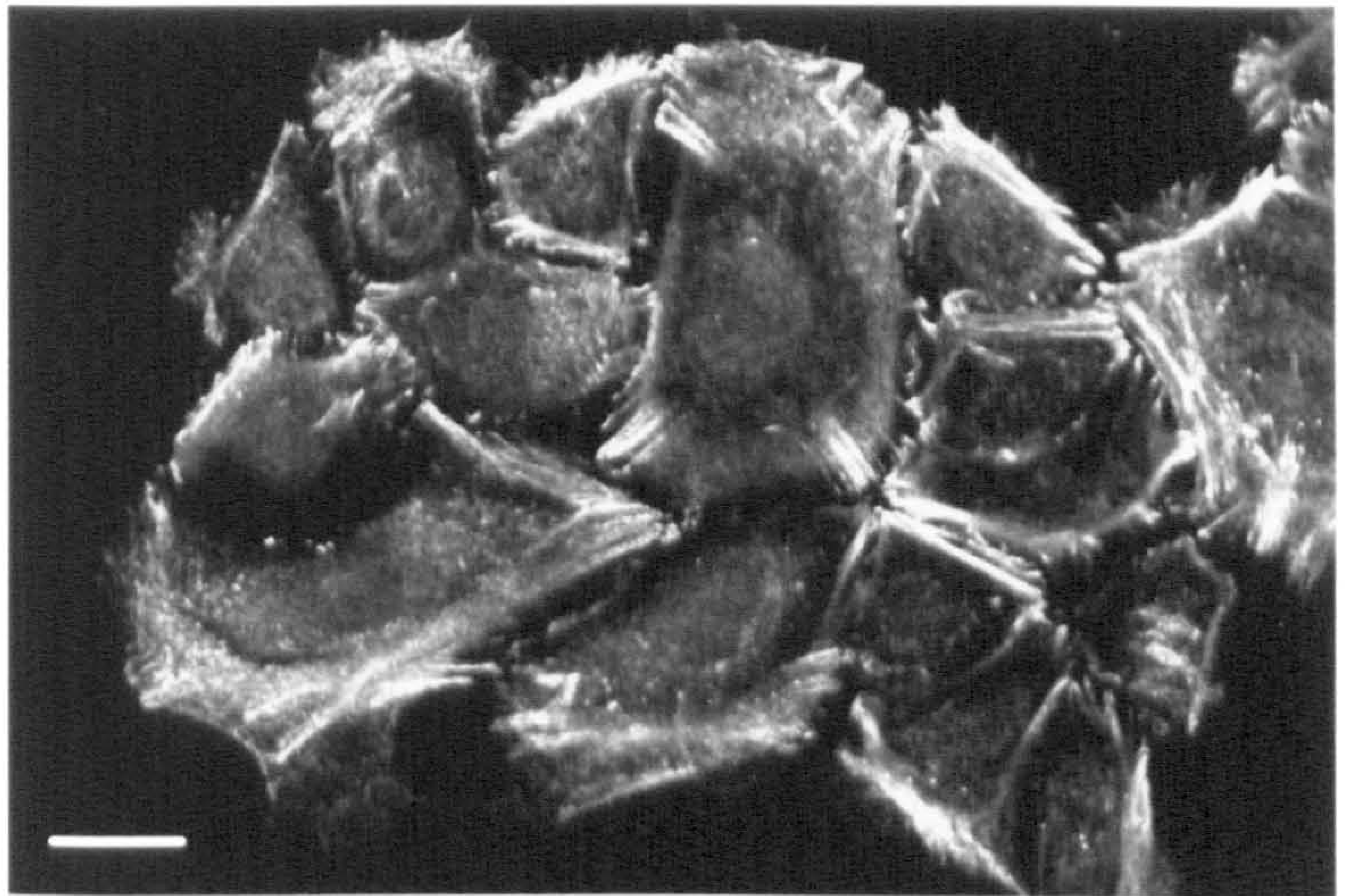
Confocal immunofluorescence micrographs of adherent keratinocytes stained for actin after treatment with 0.1% DMSO (*a*), 2.5μg/ml nocodazole (*b*) or 5μg/ml cytochalasin D (*c*) for 4½ hours. The antiserum used was anti-actin mAb AC 40 diluted 1:100 and visualised with anti-mouse-FITC diluted 1:100. Scale bars represent 25μm.

Cytochalasin D-induced actin disruption

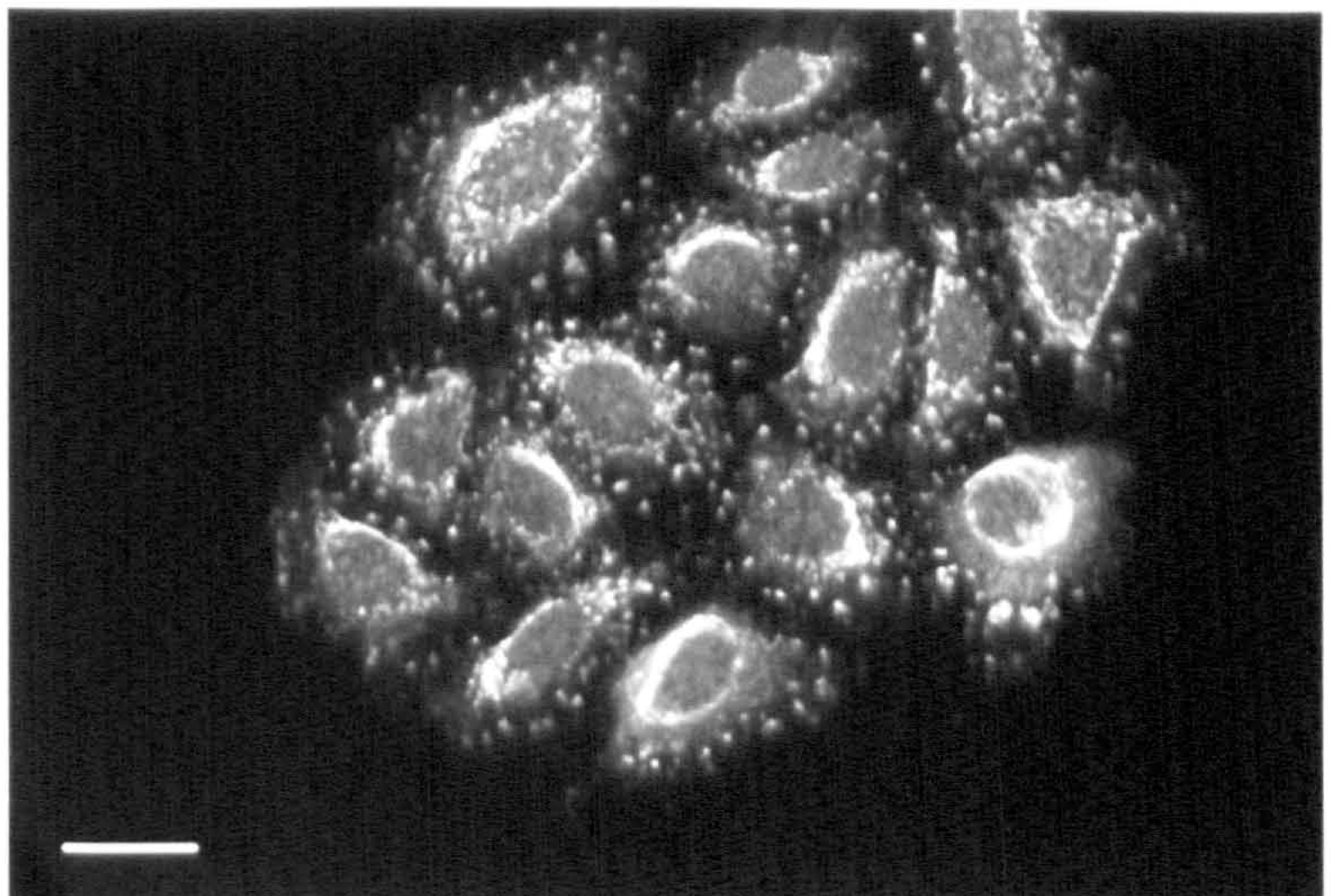
a. low extracellular calcium, no drug



b. low extracellular calcium, nocodazole for 4 hours



c. low extracellular calcium, cytochalasin D for 4 hours



Cytochalasin D treatment did not totally prevent the accumulation of E- and P-cadherin at areas of cell-cell contact - punctate staining for both cadherins was visible in the cell-cell adhesions of treated keratinocytes (Figure 16 *d* and 17 *d*). Cytochalasin D did not depolymerise the microtubules or the K14-containing intermediate filaments although the organisation of these cytoskeletal structures was altered, possibly a consequence of the impaired cell-cell adhesion in these cells (Figures 18 *c* and 20 *c* - the remainder of Figure 20 is described in section 6.5.3). Thus, the Ca^{++}-dependent translocation of E- and P- cadherin to areas of cell-cell contact was inhibited by treatment with cytochalasin D but not nocodazole. This suggests that the cadherin relocalisation process is actin- (but not microtubule) dependent.

B. Abundance of the classical cadherins

In addition to the subcellular localisation studies, the abundance of E- and P-cadherin in adherent keratinocytes grown in low extracellular Ca^{++} and after switching to high extracellular Ca^{++} was examined by immunoblotting (Figure 21). In subconfluent keratinocytes maintained in LKGM (t = 0), both cadherins were readily detectable but the P-cadherin antiserum reacted with three proteins of different electrophoretic mobility, which appeared to be present in comparable amounts (Figure 21 *b*). Switching to HKGM did not result in any changes in the amount of E-cadherin present (Figure 21 *a*), but the pattern of anti-P-cadherin reactive proteins was altered. The upper two species which were detected by the anti-P-cadherin antiserum in proteins extracted from cells grown in LKGM were no longer visible and the lower species appeared stronger. However, no further changes were observed in the amount of P-cadherin present with prolonged incubation in HKGM. See section 6.5.2 for a discussion of Figure 21 *d*.

Immunoblotting for the cadherin-associated protein β-catenin, revealed that there were no significant changes in the total amounts of this protein present in response to high extracellular Ca^{++} (Figure 21 *c*). Furthermore, no changes in the electrophoretic mobility of β-catenin were induced by switching to HKGM (the t = 0 lane on the immunoblot appears to have a slightly lower mobility due to its proximity to the edge of the polyacrylamide gel during electrophoresis). Thus, switching to HKGM, alters the electrophoretic mobility of P-cadherin but not E-cadherin or β-catenin. This may be indicative of some form of post-translational processing of P-cadherin in response to high extracellular calcium.

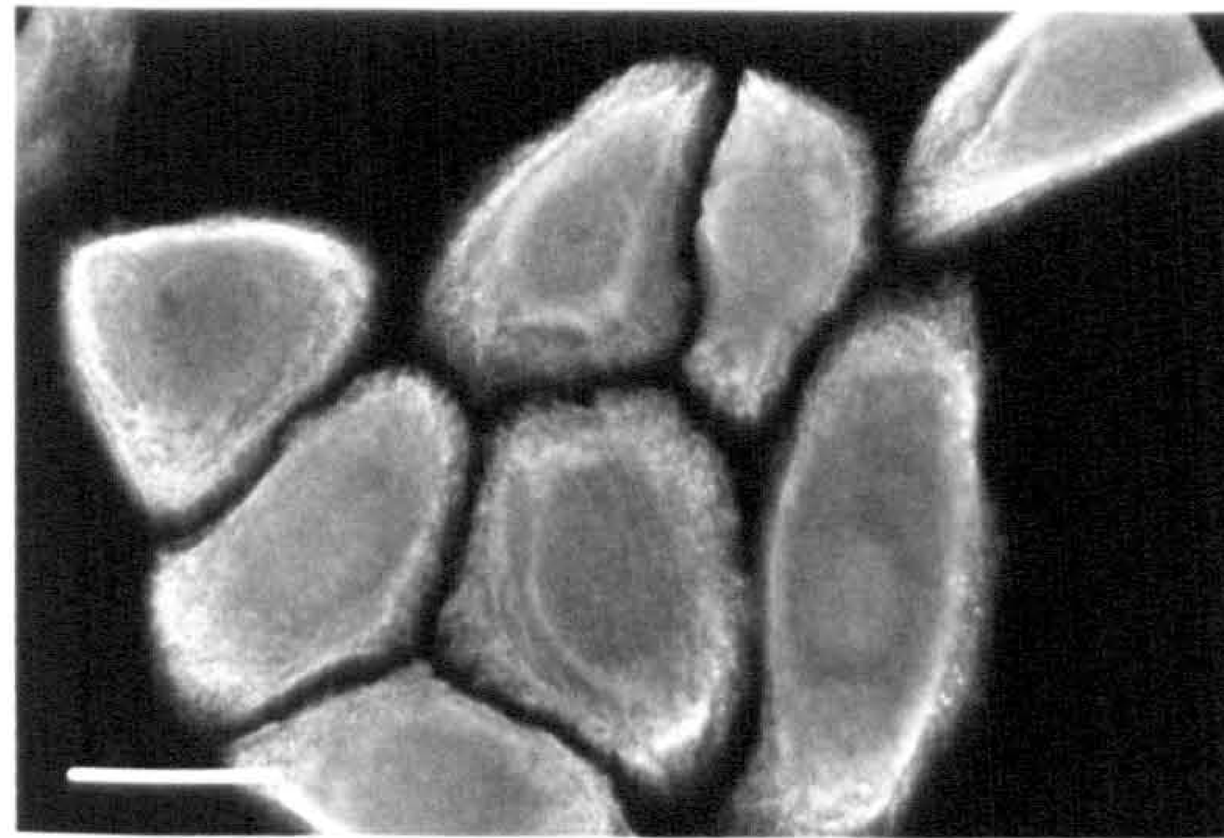
Figure 20

Figure 20
Keratin 14 distribution and abundance in low and high extracellular calcium

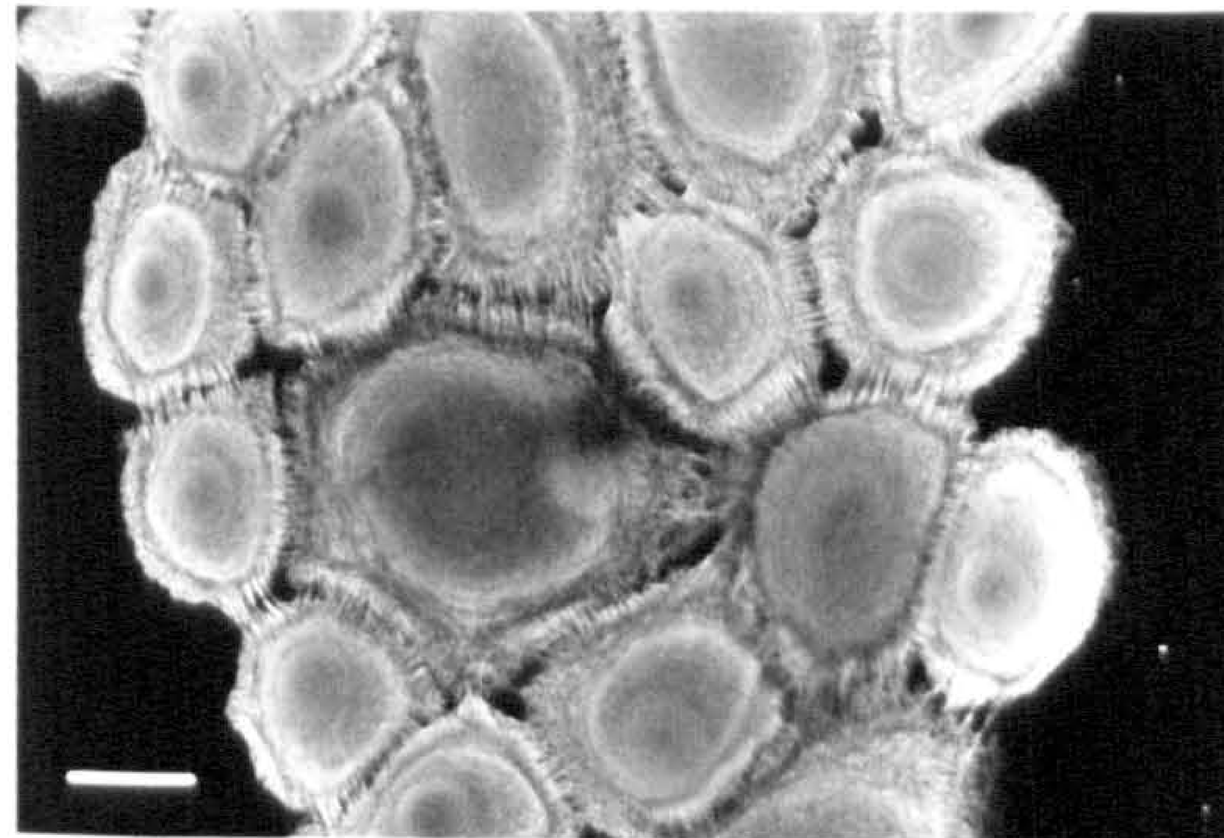
Confocal immunofluorescence micrographs of adherent keratinocytes stained for K14 after growth in 0.03mM extracellular Ca^{++} (*a*), after growth in 1mM extracellular Ca^{++} for 4 hours (*b*), after incubation in 1mM extracellular Ca^{++} for 4 hours in the presence of 5μg/ml cytochalasin D (*c*) and after incubation in 1mM extracellular Ca^{++} in the presence of 2.5μg/ml nocodazole (*d*). Panel *e*. shows an immunoblot for K14 prepared using proteins extracted using RIPA buffer from cells grown in 0.03mM Ca^{++} (t = 0) and shifted to 1mM extracellular Ca^{++} for the times indicated. The antiserum used was mAb LL001 (a gift from Professor. E.B. Lane, University of Dundee) diluted 1:100 for immunofluorescence and 1:1000 for immunoblotting. Scale bars represent 25μm.

K14 distribution and abundance in low and high extracellular calcium

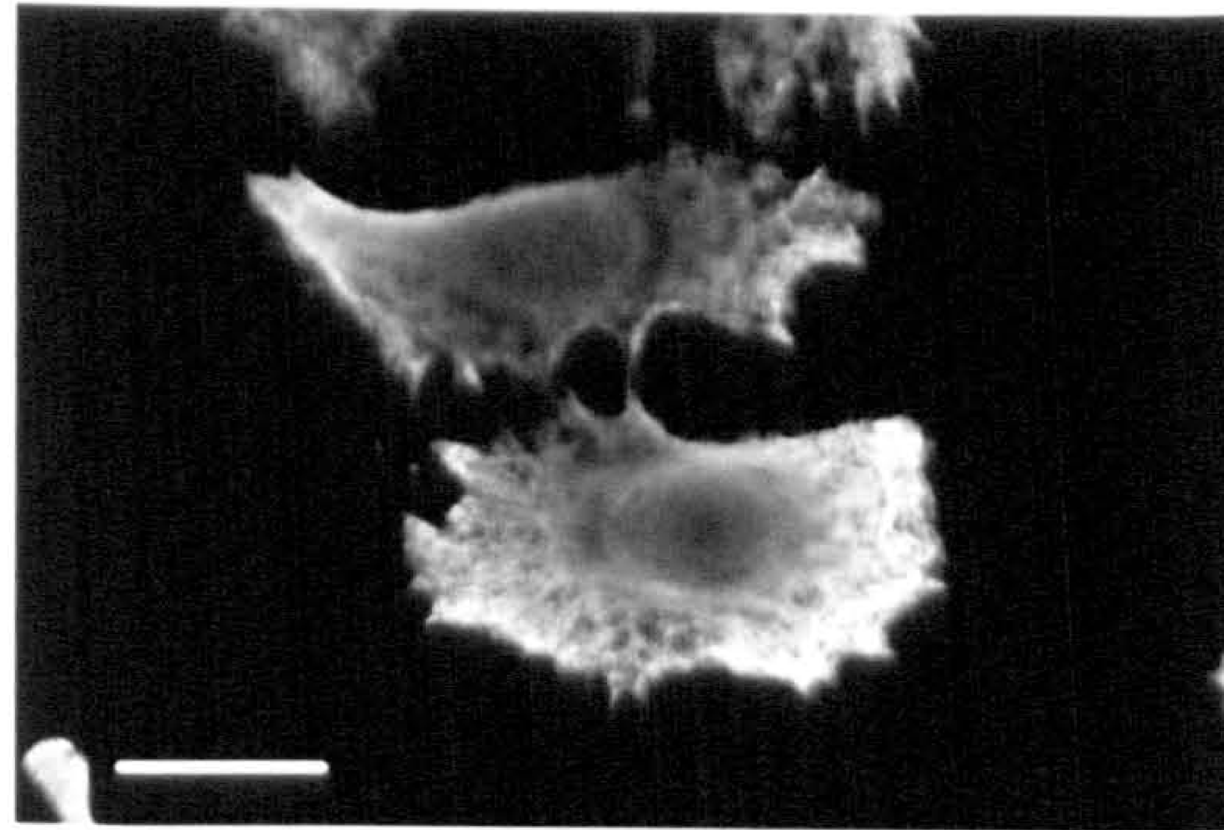
a. low extracellular calcium



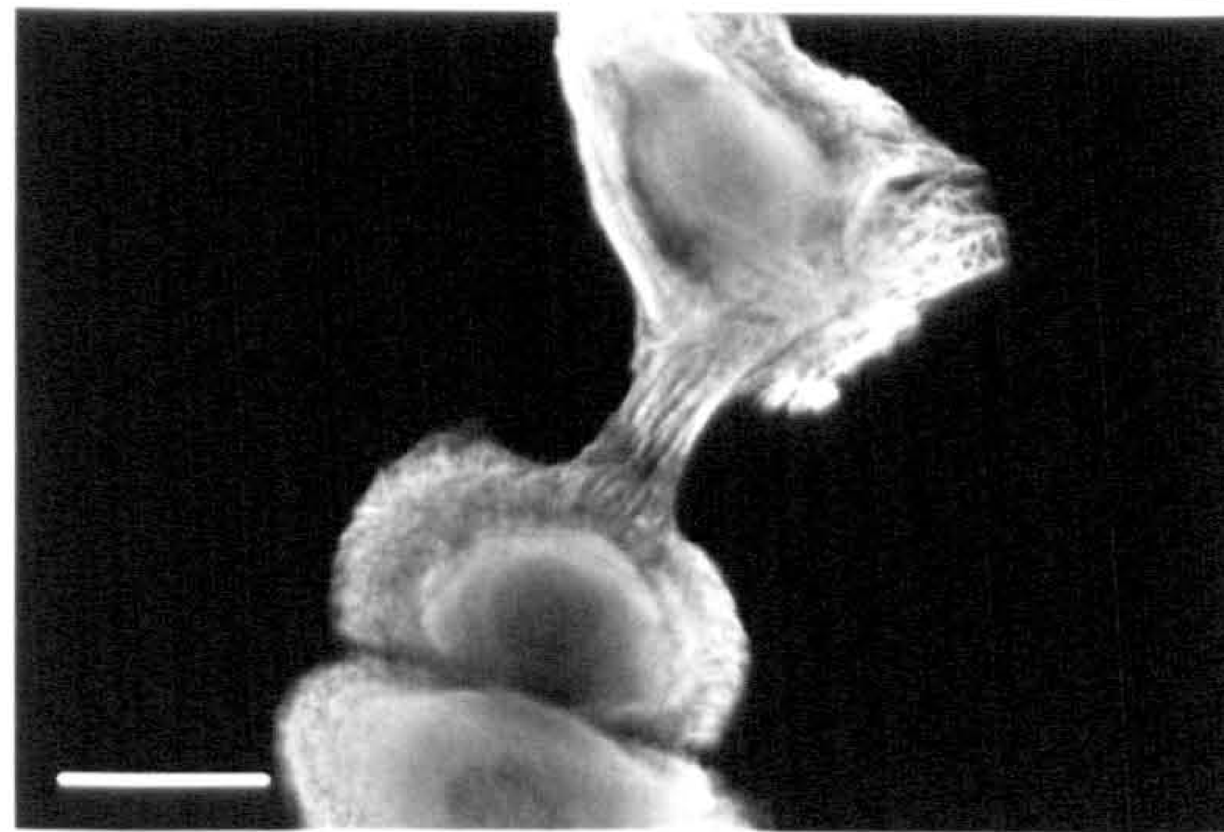
b. high extracellular calcium, 4 hours



c. high extracellular calcium, 4 hours + cytochalasin D



d. high extracellular calcium, 4 hours + nocodazole



e. K14 immunoblot

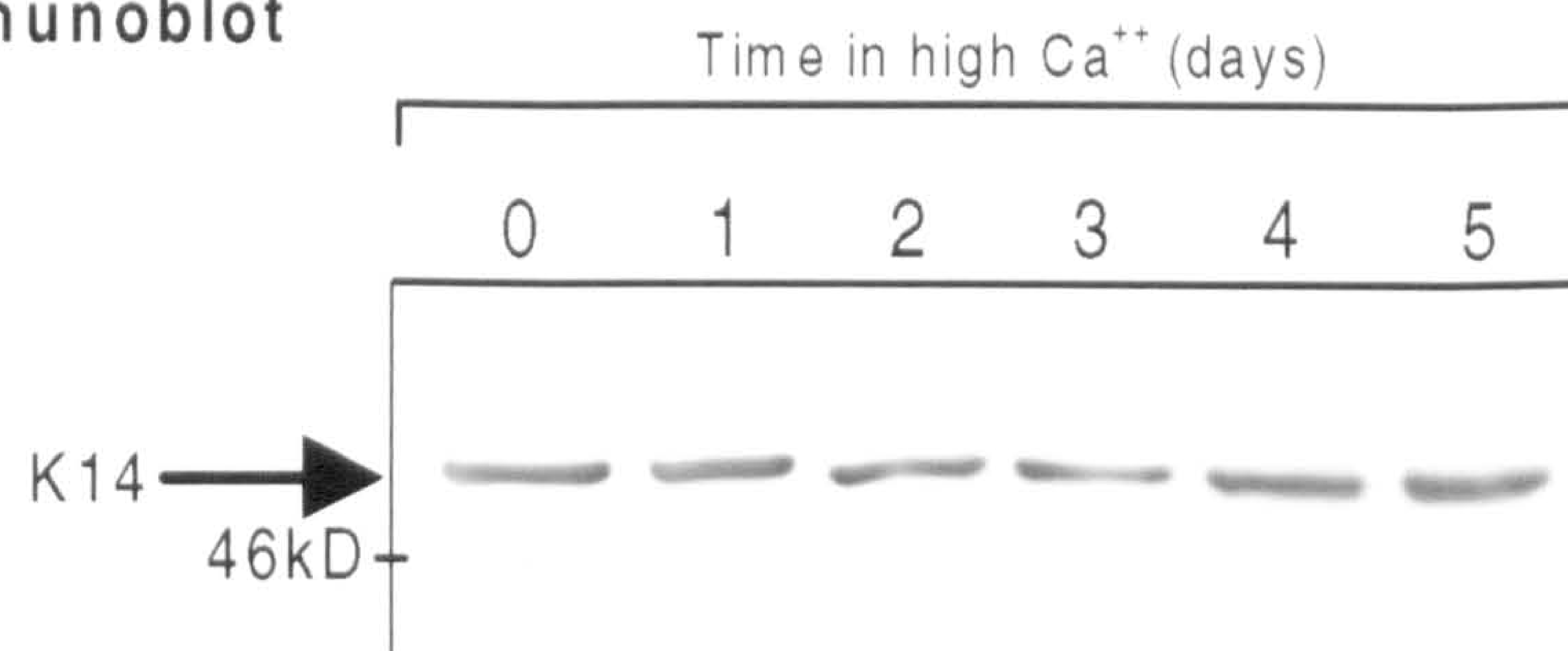


Figure 21

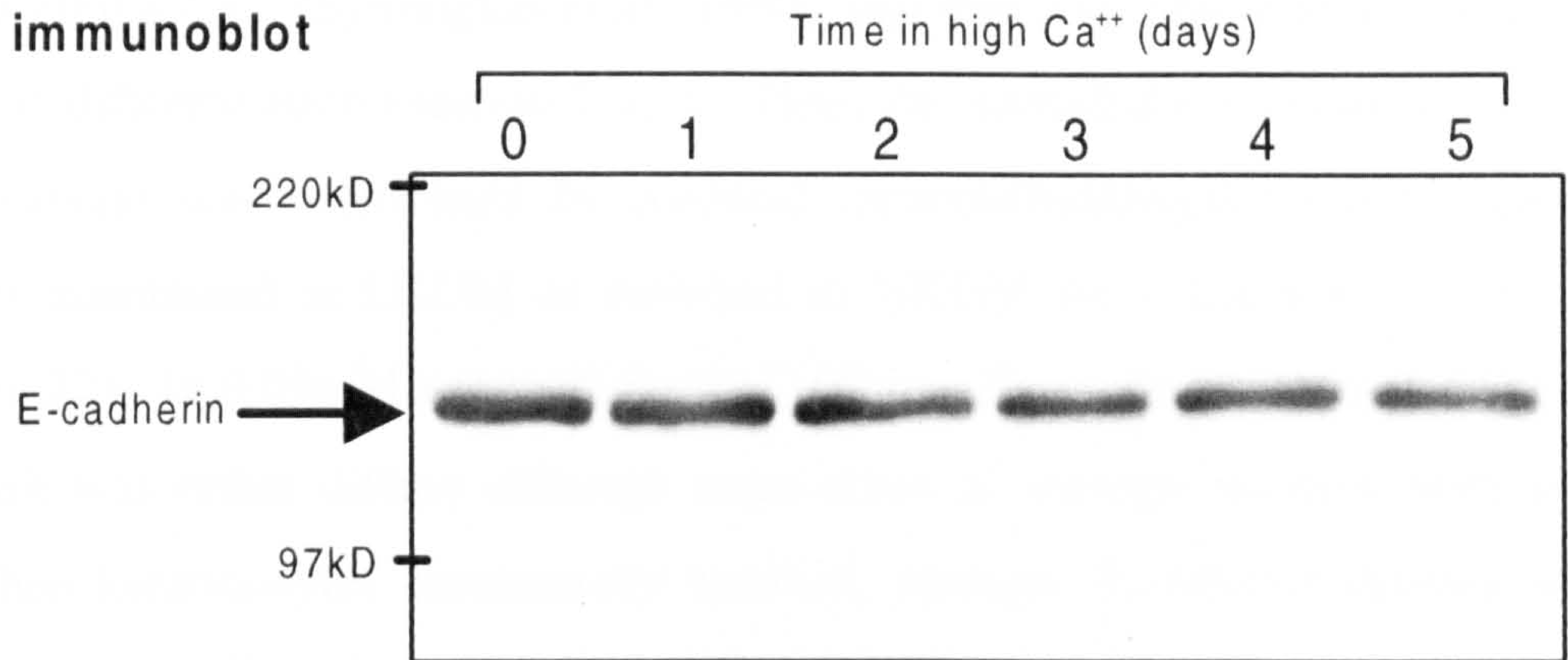
Figure 21

The effects of extracellular calcium on cadherin, catenin and integrin abundance

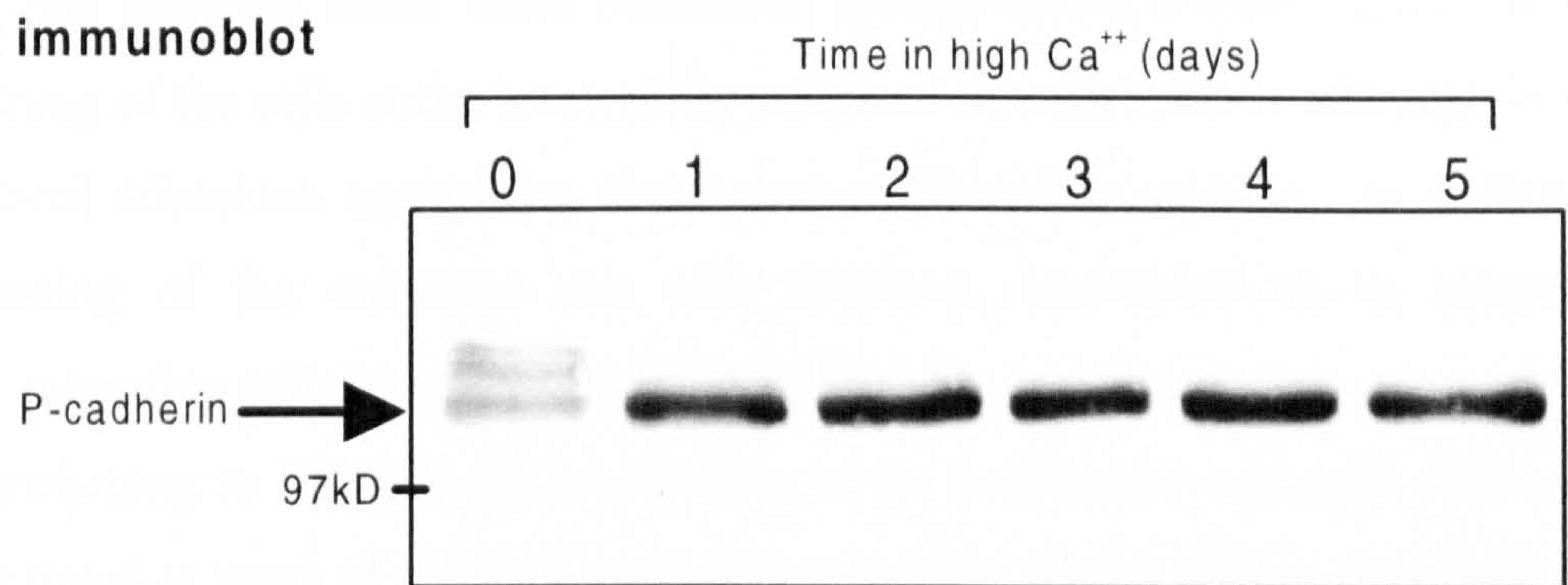
Immunoblots for E-cadherin (*a*), P-cadherin (*b*) β-catenin (*c*) and β_1-integrin (*d*) prepared with proteins extracted using RIPA buffer (section 4.11.2) from adherent keratinocytes grown in 0.03mM Ca^{++} (t = 0) or switched to 1mM extracellular Ca^{++} for a duration of up to 5 days. Molecular weight markers are indicated. The antisera used were mouse anti-E-cadherin (clone #36) diluted 1:2000, mouse anti-P-cadherin (clone #56) diluted 1:500, rabbit polyclonal anti-β-catenin diluted 1:2000 and rat anti-β_1-integrin (mAb13) diluted 1:1000.

The effects of extracellular calcium on cadherin, catenin and integrin abundance

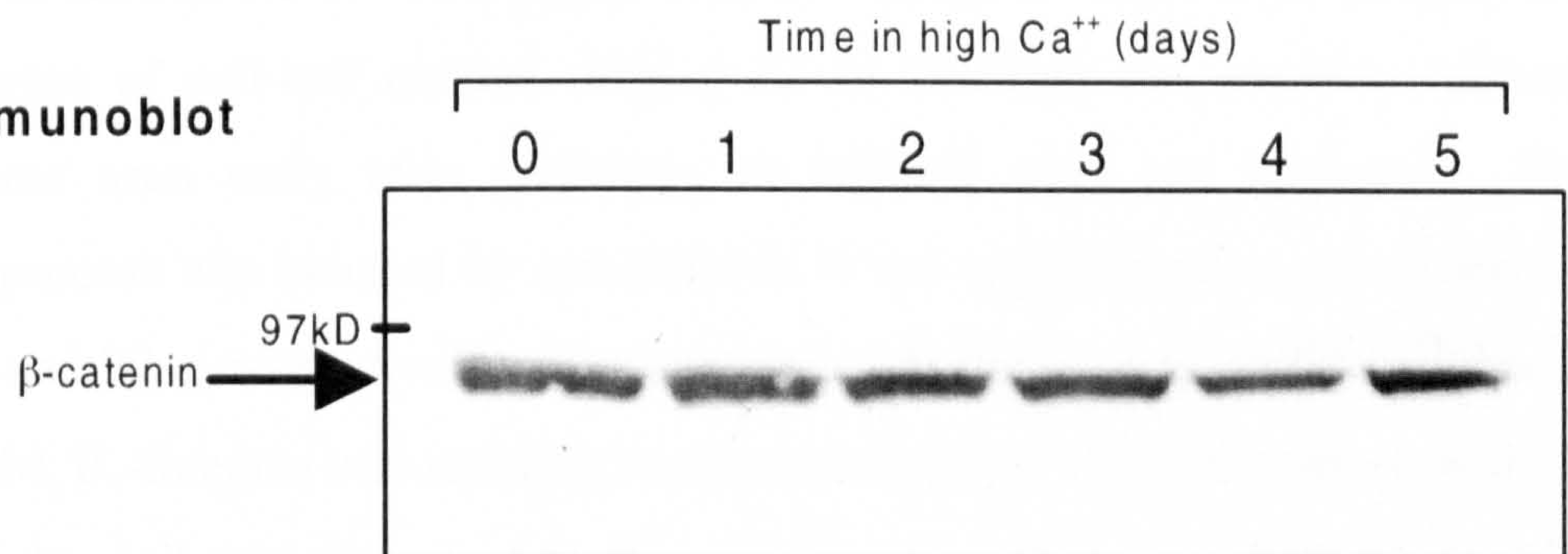
***a.* E-cadherin immunoblot**



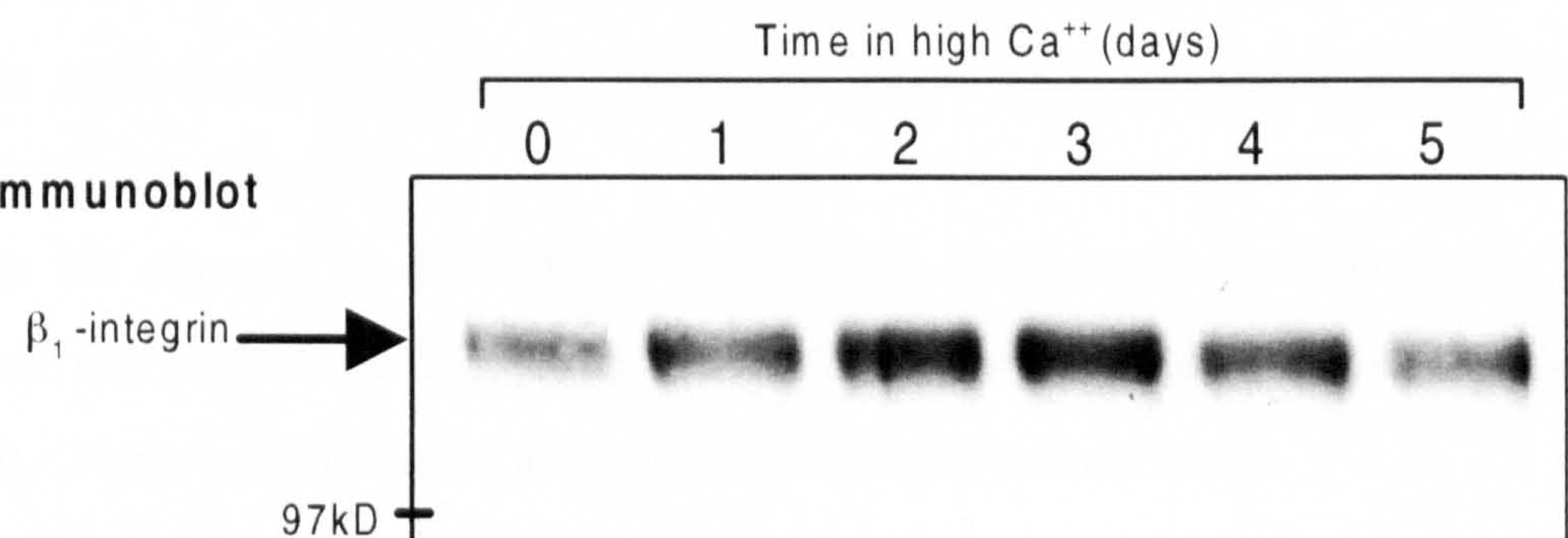
***b.* P-cadherin immunoblot**



***c.* β-catenin immunoblot**



***d.* β_1-integrin immunoblot**



6.5.2 High extracellular calcium induces the relocalisation of β_1-integrin to cell-cell contacts

There is some evidence that the integrins play a role in keratinocyte intercellular adhesion, particularly where the formation of cadherin-mediated junctions is suppressed by low extracellular Ca^{++} (Symington *et al.,* 1993), and they may also play a role in the modulation of differentiation (section 2.1.2). Thus, the subcellular localisation of the β_1-integrin subunit was determined by confocal immunofluorescence microscopy in keratinocytes maintained in LKGM or switched to HKGM for a duration of 4 or 24 hours (Figure 22). In 0.03mM extracellular Ca^{++} (Figure 22 *a*), the staining pattern for the β_1-subunit was rather diffuse although some areas of stronger staining were also visible. When keratinocytes occasionally touched, stronger β_1-subunit staining was apparent in the cell-cell contact area, suggesting that this integrin may participate in a form of cell-cell adhesion under these conditions (closed arrow, Figure 22 *a*). Some punctate staining of the cells at the level of the substrate was also visible although in no cells were focal adhesions resembling those seen in fibroblasts visible. In addition, punctate staining of the substrate was also apparent, corresponding to integrins deposited by migrating cells (open arrow, Figure 22 *a*).

After switching to HKGM, the localisation of the β_1-integrin was quite different, being concentrated at areas of cell-cell contact in a manner similar to that described for the cadherins in section 6.5.1. Four hours after switching to HKGM, β_1-integrin was localised to areas of cell-cell contact (Figure 22 *b*) although the complex cadherin staining patterns seen early after switching to HKGM were not apparent. This relocalisation process was blocked by cytochalasin D but was insensitive to nocodazole (Figures 22 *d* and 22 *e*, respectively), in agreement with Braga *et al.* (1995). After 24 hours in HKGM, β_1-integrin was strongly localised to areas of cell-cell contact, with the remainder of the cell staining weakly (Figure 22 *c*). Thus, in response to high extracellular Ca^{++}, the β_1-integrin subunit was relocalised to the areas of cell-cell contact in an actin-dependent manner, indicating that it may participate in calcium-induced intercellular adhesion.

Despite the changes in subcellular localisation, there were no changes in the electrophoretic mobility of the β_1-integrin subunit in response to high extracellular Ca^{++} (Figure 21 *d*). There was, however, a small increase in the protein levels.

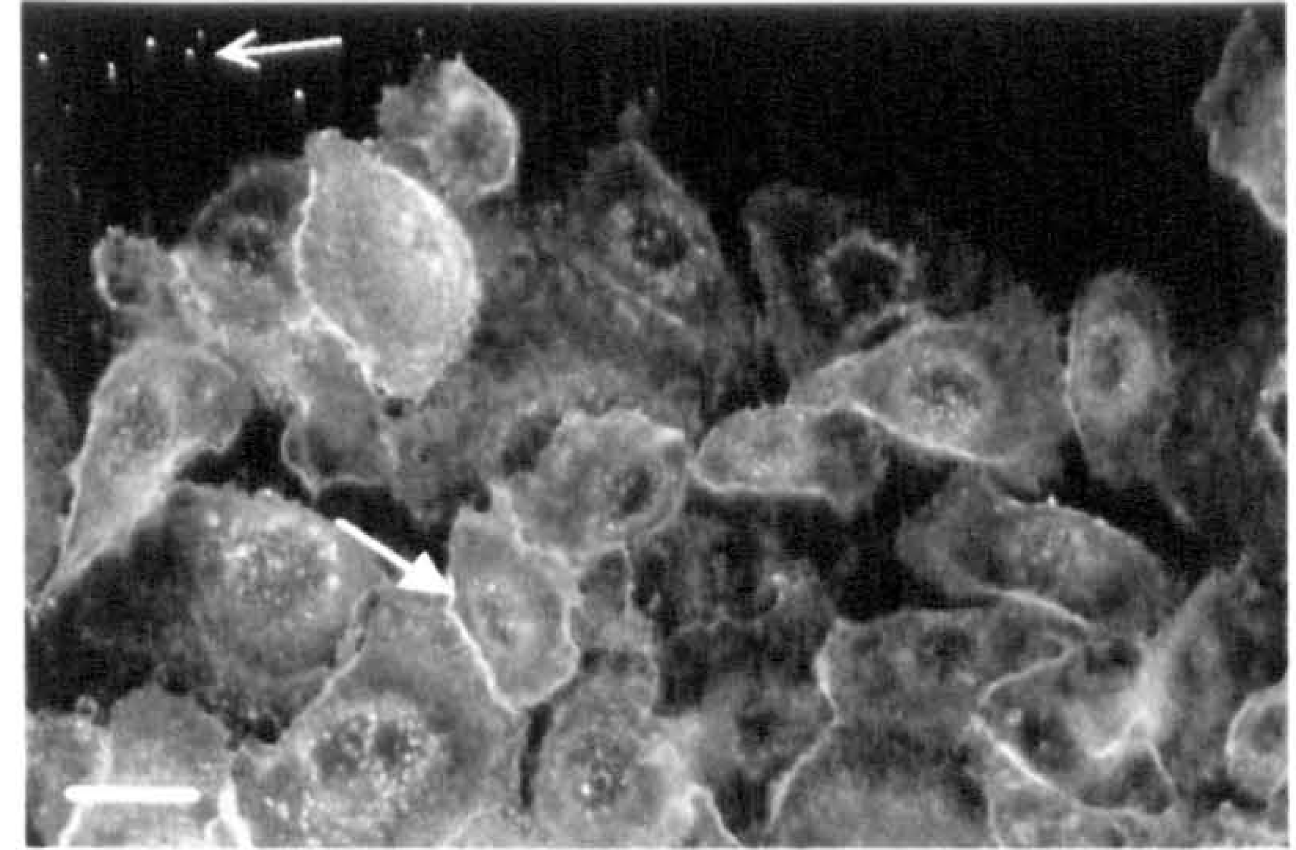
Figure 22

Figure 22
The effects of extracellular calcium on β_1-integrin localisation

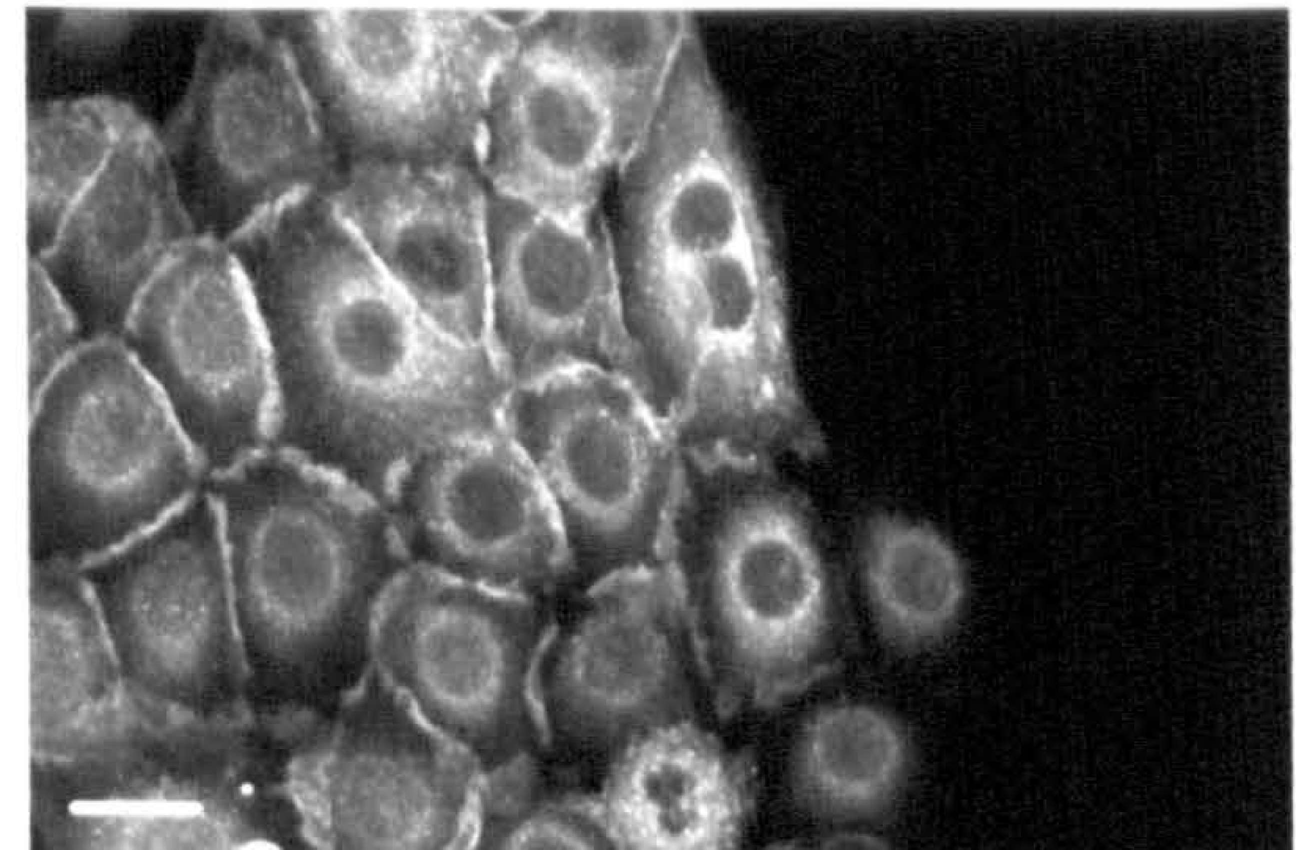
Confocal immunofluorescence micrographs showing adherent keratinocytes grown in 0.03mM extracellular Ca^{++} (*a*), after switching to 1mM extracellular Ca^{++} for 4 hours (*b*), 24 hours (*c*), 4 hours in the presence of 5μg/ml cytochalasin D (*d*) and 4 hours in the presence of 2.5μg/ml nocodazole (*e*). The antiserum used was mAb 13 (rat anti-β_1-integrin) diluted 1:100 visualised with anti-rat-FITC diluted 1:125. Scale bars represent 25μm.

The effects of extracellular calcium on β_1-integrin localisation

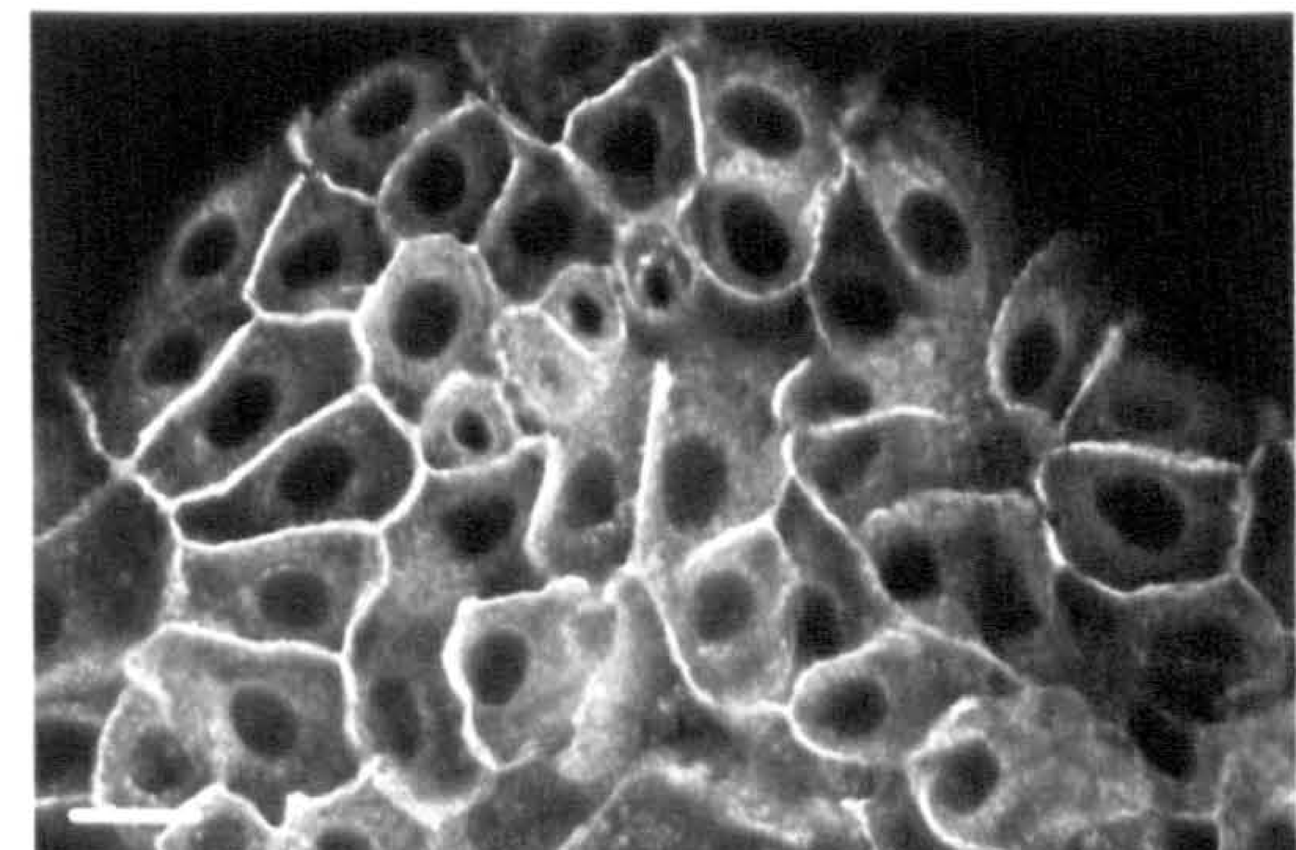
a. low extracellular calcium



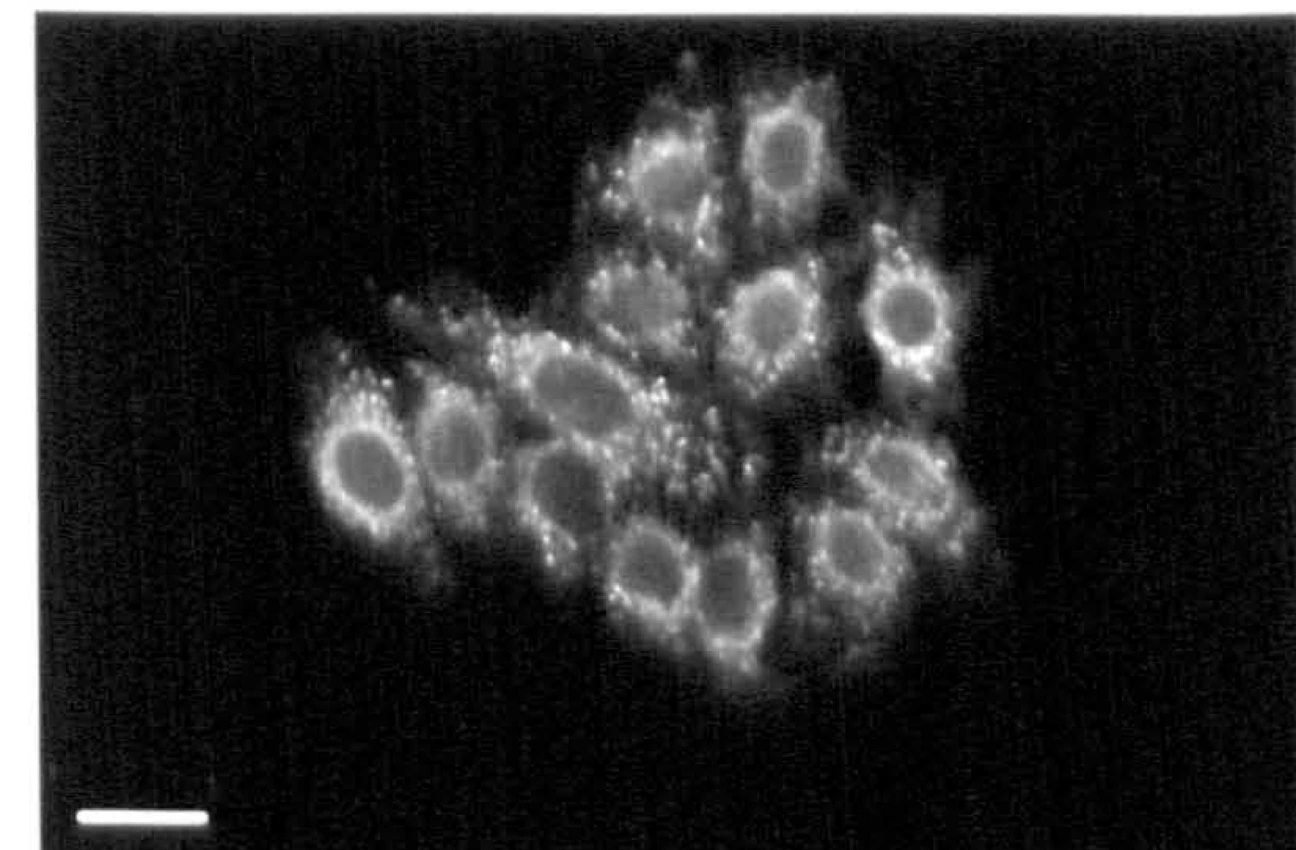
b. high extracellular calcium, 4 hours



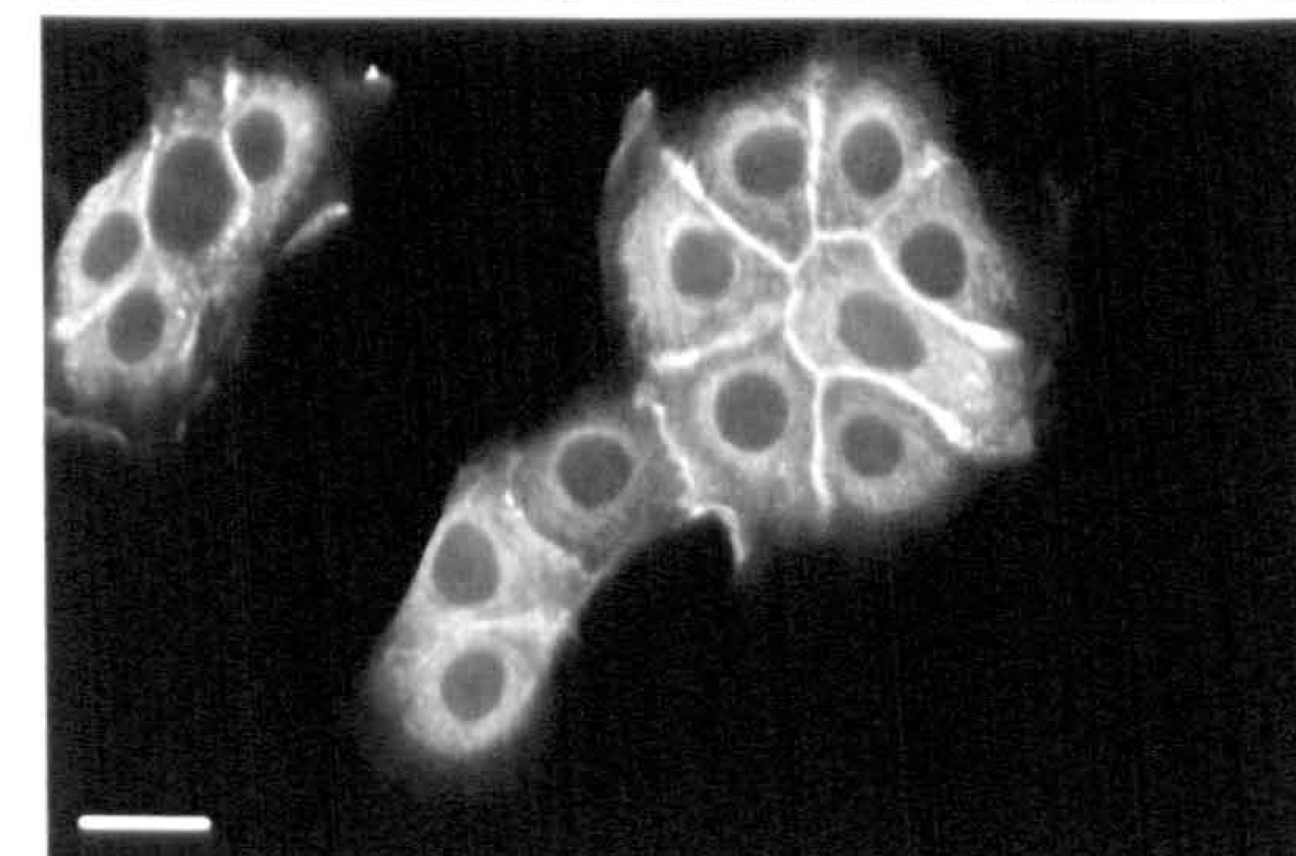
c. high extracellular calcium, 24 hours



d. high extracellular calcium and cytochalasin D for 4 hours



e. high extracellular calcium and nocodazole for 4 hours



of this subunit up to 3 days after switch to HKGM, which then declined again, back to the basal level after 5 days in HKGM.

6.5.3 High extracellular calcium induces changes consistent with desmosome assembly

The subcellular distribution of desmosomal components was not investigated during this study. However, immunofluorescent staining for keratin K14 revealed dramatic changes in the intermediate filament organisation, which are consistent with the assembly of desmosomes (Figure 20). In low extracellular Ca^{++}, the K14-containing intermediate filaments appeared relatively disorganised although more prominent filaments extending along some cells were visible (Figure 20 *a*). Soon after switching to HKGM, the K14-containing intermediate filaments rapidly reorganised and inserted into areas of cell-cell contact, giving the cells a very distinctive appearance (Figure 20 *b*). Furthermore, this arrangement of keratin was not seen on the free edges of cells suggesting that it was specific to cell-cell adhesions. The staining pattern described for E-cadherin in section 6.5.1A, where the cadherin protein appeared to be inserting into sites of cell-cell adhesion in a series of fibres, suggests that the cadherins could colocalise with the reorganised K14-containing intermediate filaments. This implies that the formation of desmosomes may occur where adherens junctions are forming or that the keratins are involved in the formation of adherens junctions in some way. This process of intermediate filament reorganisation was not affected by nocodazole (Figure 20 *d*) but was altered by cytochalasin D (Figure 20 *c*). In HKGM, in the presence of cytochalasin D, the K14-containing intermediate filaments were visible in the cells, indicating that the drug did not cause the degradation of these cytoskeletal structures. However, the organisation of the filaments was perturbed, probably because of the absence of normal intercellular adhesion under these conditions.

Despite the profound changes in K14 arrangement that occurred in response to high extracellular Ca^{++}, no changes were seen in the electrophoretic mobility or protein levels of K14 after switching to HKGM (Figure 20 *e*), as determined by immunoblotting. Even after 5 days in HKGM, when many cells were differentiating, no change was seen, indicating that K14 is probably retained in these cells in high extracellular Ca^{++}. Thus, switching to HKGM causes rapid reorganisation of the K14-

containing intermediate filaments, consistent with the formation of desmosomes, but has no obvious effect on the amounts of this keratin present.

6.6 High extracellular calcium induces the reorganisation of the actin cytoskeleton

The actin cytoskeleton was clearly visible in keratinocytes maintained in LKGM stained with an anti-actin antibody with the majority of the actin concentrated around the periphery of the cells (Figure 23 *a*). More prominent fibres which appeared to terminate at the level of the substrate close to the edges of the cells were also visible, possibly corresponding to stress-fibres terminating in the focal adhesions (Carter *et al.,* 1990b). Soon after switching to HKGM, the actin distribution was quite different, with fewer fibres terminating in the cell-substrate contacts. The majority of the actin filaments remained around the periphery of the cells but mostly appeared to terminate at the sites of cell-cell contact, where filamentous actin could be seen inserting into the cell-cell adhesions (Figure 23 *b*). This subcellular localisation of actin is consistent with the physical interactions which occur between the classical cadherins and the actin cytoskeleton (Ozawa *et al.,* 1989). These observations are consistent with those where keratinocytes were stained specifically for filamentous actin (Kubler *et al.,* 1991), indicating that the amounts of globular actin present in these cells do not interfere with this analysis.

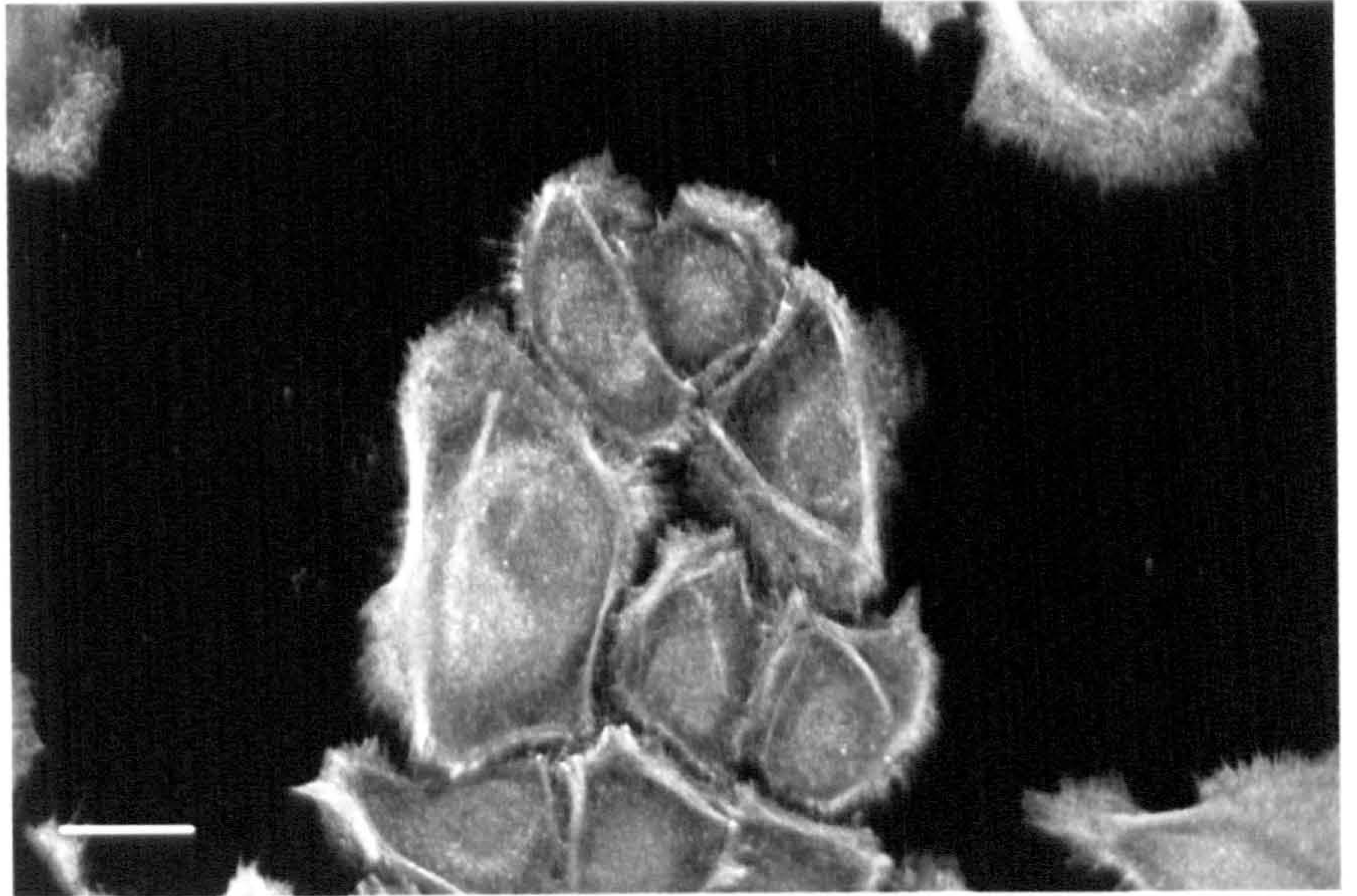
Figure 23

Figure 23
Calcium-induced actin reorganisation

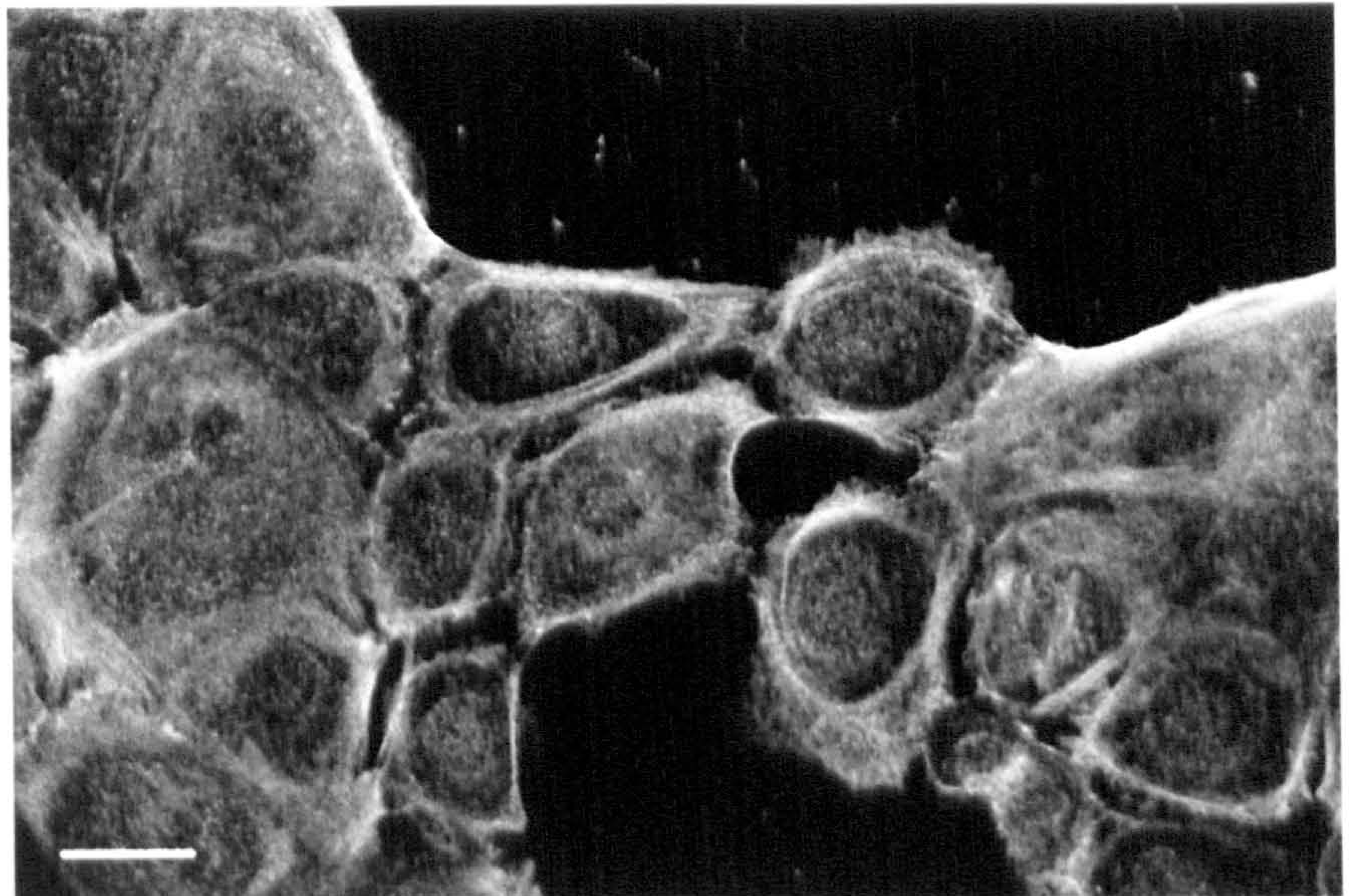
Confocal immunofluorescence micrographs of adherent keratinocytes stained for actin after maintaining in 0.03mM extracellular Ca^{++} (*a*) and after switching to 1mM extracellular Ca^{++} for 4 hours (*b*).

Calcium-induced actin reorganisation

a. low calcium



b. high calcium,
4 hours



6.7 DISCUSSION

The purpose of the experiments described in this chapter was to evaluate the behaviour of keratinocytes in the MCDB 153 culture system and to consider the early events induced by high extracellular Ca^{++} which might initiate differentiation. The data presented in this chapter indicate that the formation of cell-cell contacts precedes the expression of differentiation-specific proteins and that the cadherins and integrins are candidate mediators of junction formation.

6.7.1 High extracellular calcium induces responses characteristic of *in vivo* differentiation

Switching keratinocytes from low to high extracellular Ca^{++} caused a series of changes characteristic of the *in vivo* differentiation process, including the expression of differentiation-specific proteins. The temporal differences between the beginning of increased involucrin, transglutaminase and filaggrin expression and the acquisition of peanut agglutinin binding in response to Ca^{++} partly reflect the different locations in the epidermis where these events normally first become detectable. In human epidermis, involucrin and TGk are first expressed in the spinous layer (Thacher and Rice, 1985) and are both induced soon after switch to HKGM, with TGk perhaps being induced slightly sooner (section 6.2.1). Filaggrin however, is normally first expressed in the granular layer (Lonsdale-Eccles *et al.,* 1984) and correspondingly is induced at much later times than involucrin and TGk by high extracellular Ca^{++}. In normal epidermis, basal keratinocytes do not bind peanut lectin - this function is first seen in the spinous layer (Ookusa *et al.,* 1983) where the keratinocytes have also begun to express involucrin. When cells were treated with high extracellular Ca^{++}, more cells expressed involucrin than bound peanut lectin and only involucrin-positive cells bound peanut lectin (section 6.2.2.), indicating that the acquisition of PNA binding ability is a later event than the expression of involucrin *in vitro.* Thus, the differentiation process initiated in response to Ca^{++}-shift partially recreates the spatio-temporal organisation of *in vivo* differentiation.

Cornified envelope assembly is one of the important functions that a keratinocyte completes *in vivo.* In this *in vitro* system, growing keratinocytes in high extracellular Ca^{++} causes a significant increase in the proportion of cells competent to assemble

envelopes in response to ionophore (section 6.3). This indicates that in addition to the small range of envelope components and associated proteins investigated by immunoperoxidase staining and immunoblotting, high extracellular Ca^{++} must induce the expression of a wide range of envelope components and associated enzymes. It should be noted that ionophore treatment is not a physiological stimulus for envelope assembly and it is not clear whether ionophore-induced envelopes are identical to those formed *in vivo* or not, although at least one protein which is a component of envelopes formed *in vivo* is almost certainly a component of ionophore-induced envelopes (see below).

The dramatic appearance of an anti-involucrin-reactive protein of very large molecular weight (section 6.3.2) in response to ionophore treatment indicates that involucrin is a likely component of ionophore-induced envelopes. The presence of involucrin in envelopes formed *in vitro* and *in vivo* has been shown in other ways (e.g. Robinson *et al.*, 1997; Steinert and Marekov, 1997) but the appearance of this large anti-involucrin-reactive product in response to ionophore has not been previously reported.

Overall, these data are consistent with reports showing increased involucrin protein levels and inducible transglutaminase activity in keratinocytes maintained in high extracellular Ca^{++} in comparison with cells maintained in low extracellular Ca^{++} (e.g. Pillai *et al.,* 1988a). In addition, these reports also showed that maintaining keratinocytes in high extracellular Ca^{++} significantly increases the proportion of cells competent to assemble cornified envelopes in the serum-free system (Wille *et al.,*, 1984, Pillai *et al.,* 1988a; Pillai *et al.,* 1990). However, there are also reports that commitment to differentiation in murine keratinocytes is independent of the extracellular Ca^{++} concentration (Drozdoff and Pledger, 1993). This particular study was performed on post-confluent murine keratinocytes, under conditions where differentiation is likely to be strongly induced in low extracellular Ca^{++} (see section 8.3), potentially masking the effects of high extracellular Ca^{++}. Thus, *subconfluent* human keratinocytes are induced to differentiate by high extracellular calcium concentrations.

6.7.2 Calcium-induced cadherin-mediated cell-cell adhesion

One of the changes seen in response to high extracellular Ca^{++} concentrations is the aggregation of scattered keratinocytes into areas of local confluence which subsequently become multilayered (section 6.7). These alterations in cell adhesion precede the

increased expression of differentiation-specific proteins and are therefore potential mediators of Ca^{++}-induced differentiation. Thus, cell-cell adhesion was investigated in more detail. In cultures treated with 1mM extracellular Ca^{++}, the process of stratification always took place, and in these stratified groups of cells, the differentiated cells were located in the upper layers (section 6.2.1). This suggests that perhaps the mechanism by which high extracellular Ca^{++} induces differentiation is by inducing detachment from the extracellular matrix during stratification, a hypothesis consistent with numerous reports of the induction of human keratinocyte differentiation by suspension culture in methyl-cellulose (e.g. Adams and Watt, 1989). However, by reducing the extracellular concentration from 1mM to 0.6mM, the induction of involucrin was unaffected but stratification was completely prevented over the 5 day duration of the experiment (section 6.4B). Thus, stratification is not a prerequisite for differentiation and is more likely to be a consequence of the loss of integrin function that occurs in keratinocytes upon formation of cadherin-mediated cell-cell adhesions in response to high extracellular Ca^{++} (Hodivala and Watt, 1994). All the tested extracellular Ca^{++} concentrations which induced increased involucrin expression also caused the formation of cell-cell adhesions and the subsequent aggregation of the cells into areas of local confluence. Conversely, those Ca^{++} concentrations that were insufficient to induce increased involucrin expression also failed to induce the formation of cell-cell adhesions. These data are consistent with a role for the formation of intercellular adhesions in the initiation of the differentiation program.

The formation of cadherin-mediated cell-cell adhesions was found to occur rapidly in response to elevating the extracellular Ca^{++} concentration, in agreement with Lewis *et al.*, (1994b). Observation of cells which were in the process of junction assembly revealed complex cadherin staining patterns which may be indicative of the way in which junctions form under these conditions. Four hours after switching to high extracellular Ca^{++}, the staining pattern for both E- and P-cadherin in the region of cell-cell contacts resembled a series of short filaments inserting into the sites of adhesion. Furthermore, the staining patterns on opposing cells often appeared symmetrical, indicating that each cell may contribute components of the adhesion into pre-determined sites in the membranes, possibly influenced by the adjacent cell. The similarity of the cadherin staining to the actin and K14 staining in the cell-cell contacts was striking and suggests that one or both of these cytoskeletal components may be involved in the

transport of the cadherins to sites of cell-cell adhesion It is possible that such filaments could perform a similar function to actin during the translocation of v-Src to fibroblast focal adhesions from the perinuclear region, when it is thought that intracellular transport may occur along the actin stress fibres (Fincham *et al.,* 1996).

The roles of polymerised actin and the microtubule network in the cadherin translocation process were investigated by treating the cells with cytochalasin D or nocodazole, which disrupt the actin cytoskeleton and the microtubules respectively (e.g., Tomasek and Hay, 1984). Nocodazole treatment had no effect on the redistribution of E-cadherin or P-cadherin in response to high extracellular Ca^{++} despite causing the disruption of the microtubules. Conversely, treatment with cytochalasin D suppressed the relocalisation of E- and P-cadherin to cell-cell contact zones, suggesting that an intact actin cytoskeleton is required for optimal cadherin translocation in response to high extracellular Ca^{++}. However, cadherin relocalisation was not totally blocked, indicating either that the cytoskeleton was incompletely disrupted or that there are small amounts of cadherin constitutively inserted into the membranes of keratinocytes maintained in low extracellular Ca^{++}. These cadherins would then be concentrated to sites of cell-cell adhesion in response to high extracellular Ca^{++} in an actin-independent manner, perhaps acting as a 'nucleation site' for the actin-dependent membrane insertion of more cadherin. In keratinocytes treated with cytochalasin D, no filamentous actin-containing structures were visible, suggesting that the cadherins present at cell-cell contacts in these cells were concentrated at these sites in an actin-independent manner. Furthermore, cytochalasin D did not depolymerise the K14-containing intermediate filaments, nor did it disrupt the microtubules. Thus, it is possible that the initial weak cell-cell adhesions formed by keratinocytes are mediated by cadherins constitutively present in the membrane and that these adhesions are subsequently strengthened by the actin-dependent translocation of cadherins to the nascent adhesion. A recent study showed that the rearrangement of the actin cytoskeleton is required for the stabilisation of the cadherin-mediated cell-cell adhesions between keratinocytes (Braga *et al.,* 1997). During the formation of cell-cell adhesions, actin polymerisation was shown to occur at the sites of cell-cell contact, a process that is dependent on the small GTPases Rho and Rac and is required for the establishment of the junction. Taken together, the data presented in this chapter and those of others, indicate a critical role for polymerised

actin in the assembly and maintenance of cadherin-mediated cell-cell adhesions in keratinocytes.

The alterations in the electrophoretic mobility of P-cadherin but not of E-cadherin indicate that there may be important differences in the functional regulation of these two proteins in human keratinocytes. The antisera used for immunoblotting are both raised against similar regions of the two proteins and should identify the immature and mature forms described in section 2.2.2. These data suggest that E-cadherin pre-exists predominantly in the activated mature form whereas significant amounts P-cadherin exist in the larger inactive form when the cells are maintained in low extracellular Ca^{++}. Since proteolysis of this immature form to produce the mature form normally coincides with insertion into the cell membrane (Ozawa and Kemler, 1990), one corollary is that E-cadherin may be constitutively present at higher levels in the keratinocyte membrane than P-cadherin in low extracellular Ca^{++}.

6.7.3 Calcium-induced integrin-mediated cell-cell adhesion

The cadherins were not the only type of adhesion protein which translocated to sites of cell-cell adhesion in response to high extracellular Ca^{++}. Some integrins, including the β_1-subunit and the α_2 and α_3-subunits (not shown) were found to behave similarly. This is consistent with the role proposed for the integrins in partially mediating keratinocyte intercellular adhesion (Symington *et al.*, 1993) under conditions where cadherin-mediated cell-cell adhesion is suppressed (Tenchini *et al.*, 1993). Integrin-mediated adhesions of this type may have a different influence on cell behaviour from adhesion to extracellular matrix as they are thought to involve direct interactions between integrins in the absence of the normal ECM ligand (Symington *et al.*, 1993). It is possible that the ligand with which the integrin interacts may determine the response of the cell to the formation of the adhesion. Whilst the influence this form of integrin-mediated adhesion has on cell behaviour remains unknown, this type of adhesion is present between the keratinocytes of the basal layer of the epidermis and may influence keratinocyte behaviour in that location (Peltonen *et al.*, 1989).

The loss of integrin function is a feature of keratinocyte differentiation and may contribute to the expulsion of cells committed to differentiation from the basal layer of the epidermis (Hotchin *et al.*, 1995; Watt *et al.*, 1988). However, an increase in β_1-integrin protein levels was seen in response to high extracellular Ca^{++} which may be a

consequence of the formation of integrin-mediated cell-cell adhesions. At later times, the decline in the overall abundance of the β_1-subunit may reflect the degradation of this protein in differentiating cells during the stratification process. It is important to note that immunoblotting provides no information about the function of the β_1-integrin whose function may be altered without any apparent changes in abundance in these cells.

6.7.4 Calcium-induced intermediate filament rearrangements

The rearrangement of the K14-containing intermediate filaments in response to high extracellular Ca^{++} indirectly indicates that the assembly of desmosomes, another form of intercellular adhesion, may be taking place. It is reported that the function of the classical cadherins (E- and P-cadherin in this case) is required for desmosomal assembly in this cell type (Lewis *et al.,* 1994b) and therefore, the assembly of desmosomes may be a later event than the formation of E- and P-cadherin mediated junctions.

6.8 SUMMARY

The data presented in this chapter indicate that high extracellular Ca^{++} significantly increases the proportion of differentiated keratinocytes in a subconfluent culture, and that the differentiation process induced has some similarities with *in vivo* differentiation. The responses induced by high extracellular Ca^{++} mimic the basal to spinous to granular layer transition. The granular to cornified layer transition can subsequently be approximated by treatment with ionomycin. It is also clear that the mechanism by which high extracellular Ca^{++} concentrations induces differentiation is unlikely to require the breaking of cell-matrix contacts (stratification). There is indirect evidence that a factor which may contribute to the induction of differentiation is the formation of intercellular adhesions, which precedes the expression of differentiation-specific proteins. However, alternative mechanisms for the induction of differentiation cannot be excluded at this stage The experiments described in the following two chapters investigate the induction of *in vitro* keratinocyte differentiation by high extracellular Ca^{++} concentrations in more detail.

CHAPTER 7
THE KERATINOCYTE CELL CYCLE AND DIFFERENTIATION

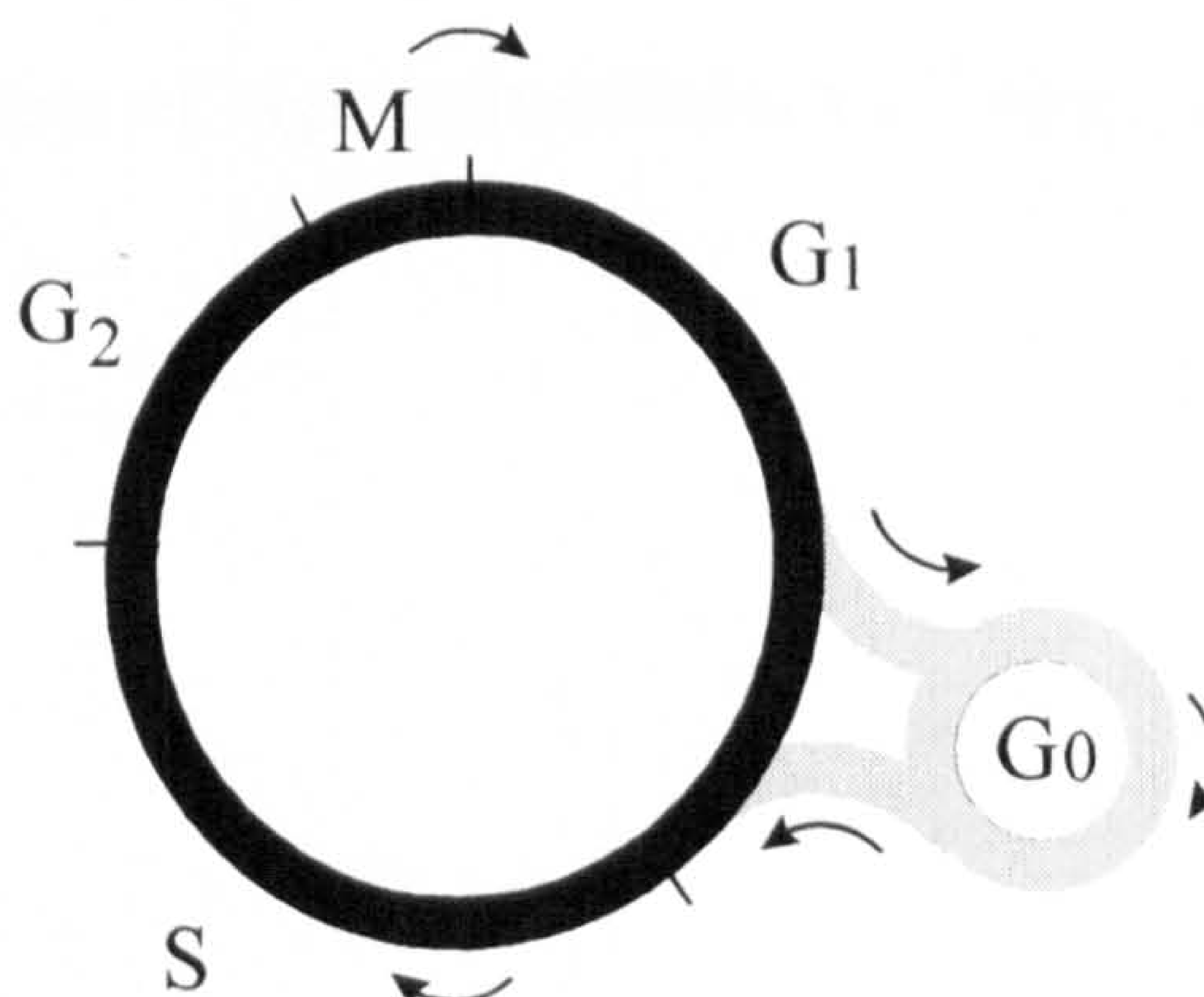
7.1 PURPOSE

The data presented in the previous chapter indicate that a potential origin of the signal which initiates differentiation in response to high extracellular Ca^{++} concentrations is the formation of intercellular adhesions. However, an alternative response to Ca^{++} which could initiate differentiation is cell cycle withdrawal, an event which precedes the expression of differentiation-specific proteins in keratinocytes deprived of cell-matrix and cell-cell contacts *in vitro* and during *in vivo* differentiation (Weinstein and Van Scott, 1965; Watt *et al.*, 1988). It has been suggested that high extracellular Ca^{++} concentrations induce murine keratinocyte differentiation indirectly, by causing irreversible growth arrest (Glick *et al.*, 1990). The purpose of the experiments described in this chapter is to examine the effects of extracellular Ca^{++} on keratinocyte cell-cycle progression and to evaluate the role these effects may play in the initiation of differentiation.

7.1.1 The cell cycle

The cell cycle, encompassing the events undergone by proliferating cells between successive divisions, is subdivided into a series of phases which are depicted in the schematic diagram below.

The cell cycle



Before division, cells are in the G_1 (1st gap)-phase of the cell cycle, which varies considerably in length according to cell type and growth conditions. This phase is followed by the S (synthetic)-phase during which the cell duplicates its DNA in preparation for division. The S-phase is followed by the second gap phase (G_2) which precedes mitosis (M), the stage where cytokinesis occurs, producing two daughter cells. Under some circumstances, cells may enter a state of reversible growth arrest, or quiescence, from the G_1 phase (Pardee, 1974). This is the G_0-phase and is represented in grey on the diagram.

7.1.2 The cell cycle and keratinocyte differentiation

In many systems, differentiation and proliferation (progress through the cell cycle) are mutually exclusive processes and the cells must cease to proliferate, often in the G_1 phase, before differentiation can begin (Scott *et al.*, 1982). Furthermore, it has been proposed that Ca^{++}-induced murine keratinocyte differentiation involves irreversible growth arrest (Glick *et al.*, 1990). The effects of extracellular Ca^{++} on keratinocyte proliferation have been reported previously with some authors reporting faster growth in high extracellular Ca^{++} (Al-Ani *et al.*, 1988; Boyce and Ham, 1983) and some reporting faster growth in low extracellular Ca^{++} (Pillai *et al.*, 1988a; Hawley-Nelson *et al.*, 1980). Some of these inconsistencies may partly result from the different techniques used to measure keratinocyte proliferation *in vitro*. In particular, analyses based on the uptake of exogenous ^{3}H-thymidine into DNA are likely to be misleading as the rate of ^{3}H-thymidine incorporation does not correlate directly with the changes in keratinocyte number (Al-Ani *et al.*, 1988; Davison *et al.*, 1979). This anomaly may be a consequence of keratinocytes utilising the thymidine salvage pathway variably (Clausen *et al.*, 1983) and results in an underestimation of the proliferation rate measured by thymidine uptake in the presence of high extracellular Ca^{++} (e.g., Pillai *et al.*, 1988a).

7.2 THE EFFECTS OF CALCIUM ON KERATINOCYTE PROLIFERATION AND DETACHMENT

7.2.1 Keratinocyte proliferation is unaffected by high extracellular calcium

To investigate the effects of Ca^{++} on keratinocyte proliferation, cells were seeded in LKGM. After 24 hours the medium was replaced either with fresh LKGM or with HKGM and was replaced daily thereafter. At daily intervals, the number of adherent cells at each Ca^{++} concentration was counted (Figure 24). These data show that there was no appreciable difference between the proliferation rates of subconfluent keratinocytes grown in 0.03mM and 1mM extracellular Ca^{++} for up to 7 days. Thus, the number of attached keratinocytes was not substantially affected by the extracellular Ca^{++} concentration.

7.2.2 Keratinocyte detachment is reduced by high extracellular calcium

Cell detachment in low and high extracellular Ca^{++} was measured so that the effect of cell loss from the monolayer on keratinocyte number could be evaluated. Cells were seeded and after 24 hours the medium was replaced with either fresh LKGM or HKGM. After four days, the number of detached and attached cells was determined (Figure 25). At no time did the cells approach confluence during these experiments. When the cells were grown in HKGM (Figure 25 *a*, red bar), there was a slightly larger number of attached cells compared with the number present after growth in LKGM (green bar), consistent with the data presented in section 7.2.1

Extracellular Ca^{++} concentration had a proportionally greater effect on the number of detached cells (Figure 25 *b*- note that the *y*-axes of chart *a* and chart *b* are plotted on different scales), with a lower rate of cell detachment in HKGM compared with LKGM. However, under both growth conditions the proportion cells of that was detached was very low, corresponding to 1% or less, of the total. This is a much smaller proportion than was reported by Pillai *et al.*, (1998a) who showed that 25% of cells maintained in low extracellular Ca^{++} were detached. However, their analyses were performed five days after the monolayer had reached confluence and therefore cannot be compared directly with the data presented here. Thus, when keratinocytes were maintained in a

Figure 24

Figure 24

The effect of extracellular calcium on keratinocyte proliferation

The number of adherent keratinocytes per $25cm^2$ tissue culture flask was monitored at daily intervals after seeding the flasks at the same initial density in LKGM and then after 24 hours (t = 0), maintaining in LKGM (green line) or switching to HKGM (red line). The cells were subconfluent at all times during this experiment.

The effect of extracellular calcium on keratinocyte proliferation

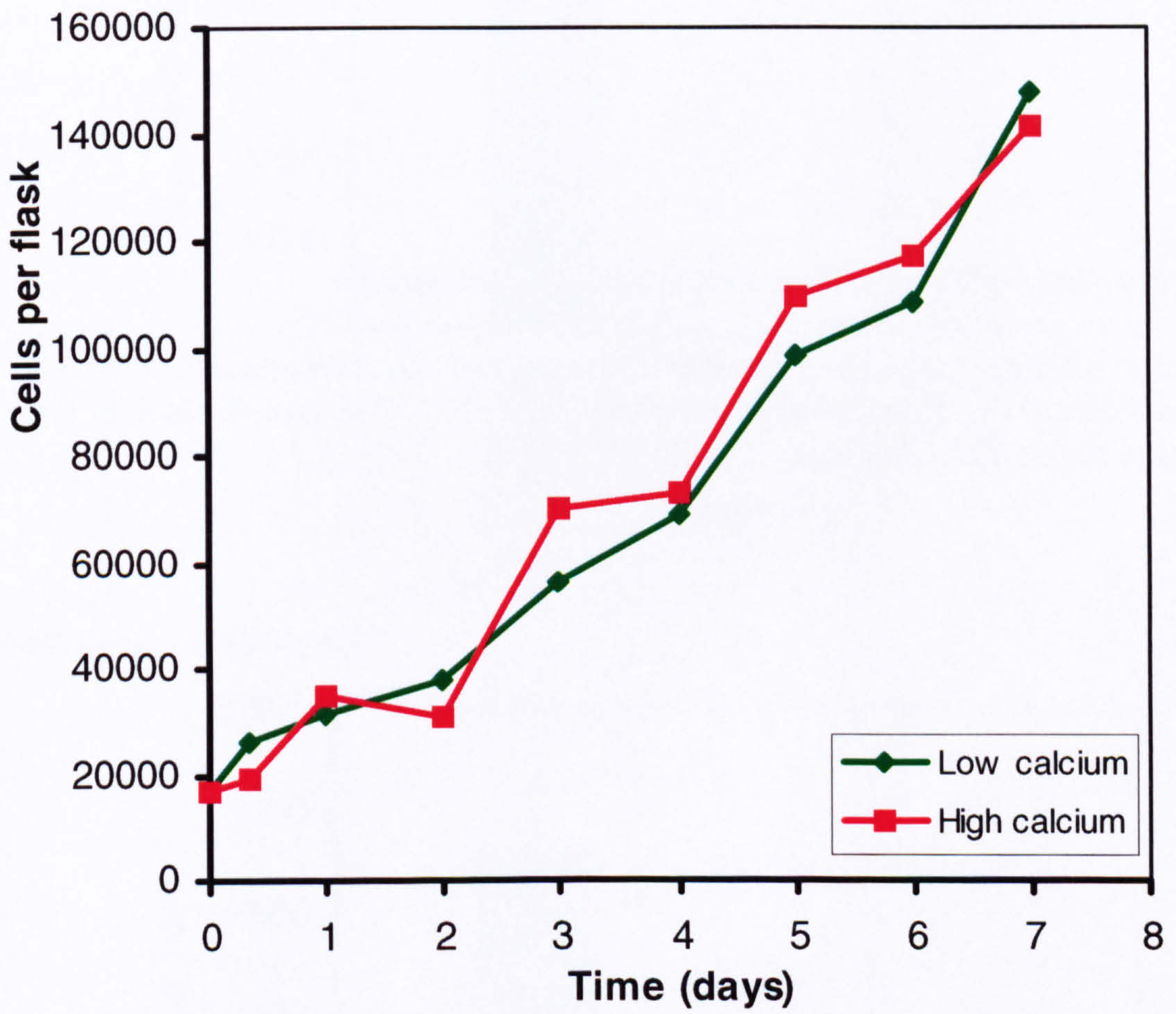


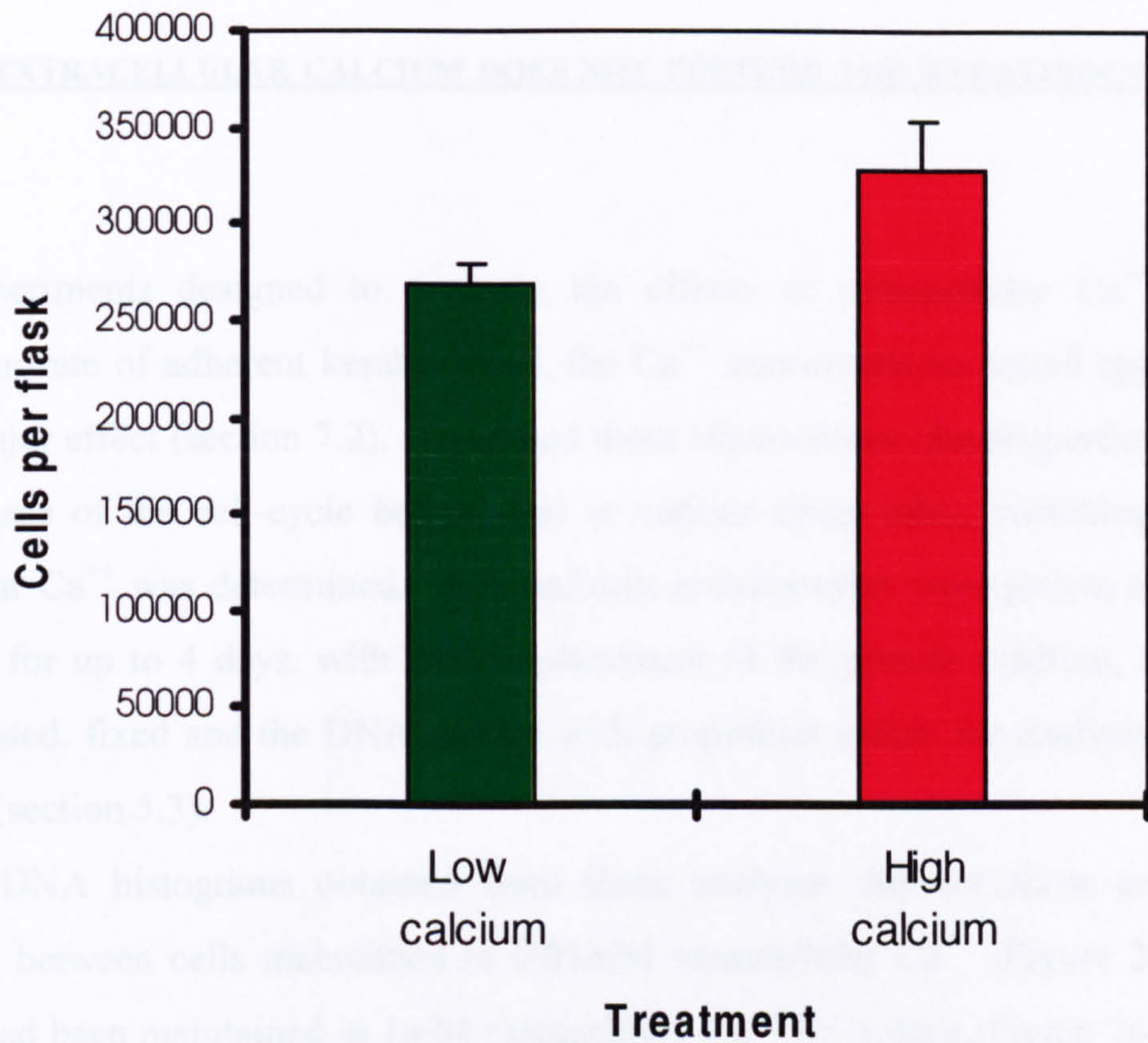
Figure 25

Figure 25
The effect of extracellular calcium on keratinocyte detachment

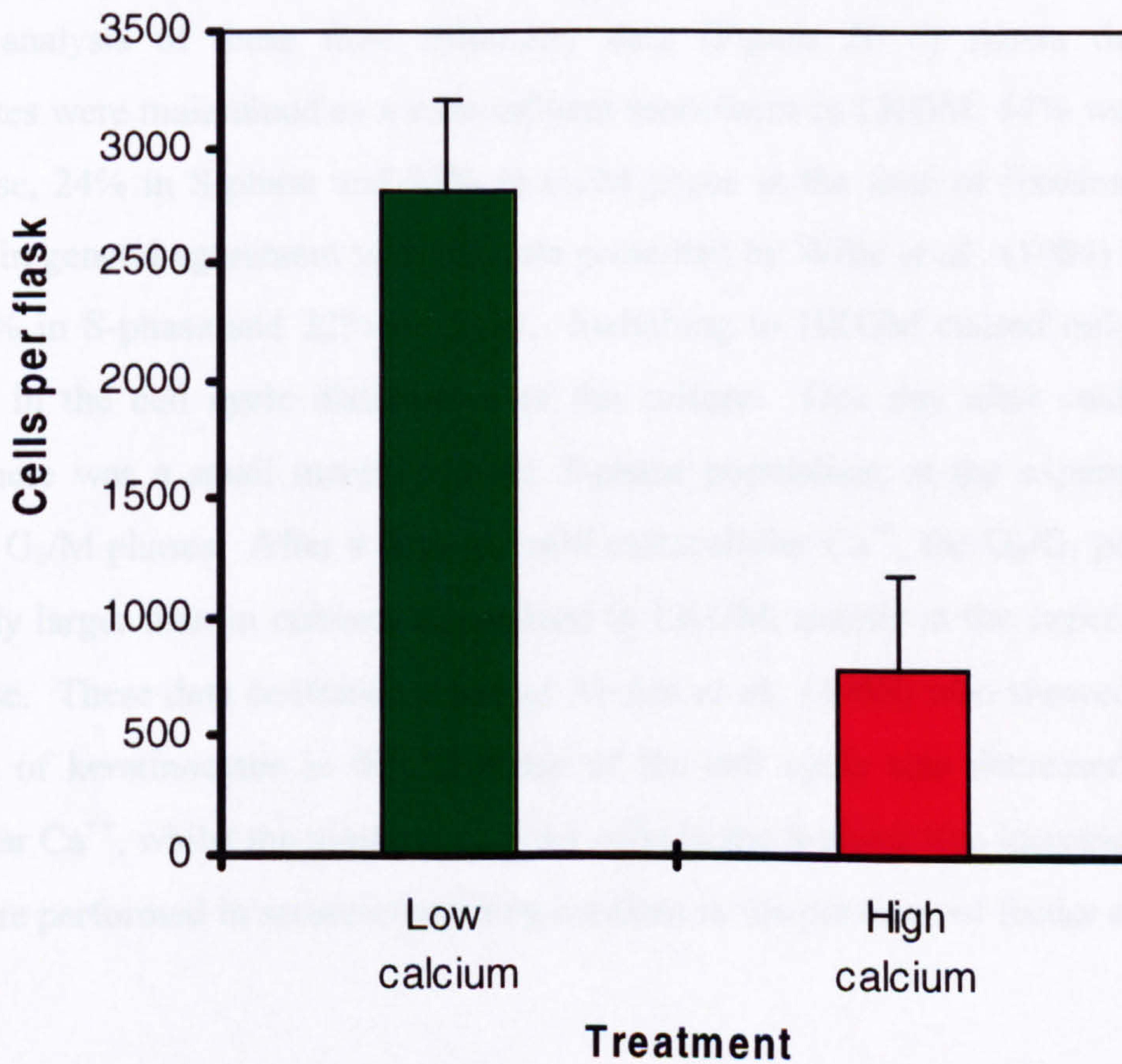
The number of adherent (*a*) and detached (*b*) keratinocytes was measured after four days growth in 0.03mM (green) or 1mM extracellular Ca^{++} (red). The growth medium was not replaced during this experiment.

The effect of extracellular calcium on keratinocyte detachment

***a.* attached keratinocytes**



***b.* detached keratinocytes**



subconfluent condition, the proportion of cells that were detached was small and was decreased still further by high extracellular Ca^{++}.

7.3 HIGH EXTRACELLULAR CALCIUM DOES NOT PERTURB THE KERATINOCYTE CELL CYCLE

In the experiments designed to evaluate the effects of extracellular Ca^{++} on the proliferation rate of adherent keratinocytes, the Ca^{++} concentrations tested appeared to have no major effect (section 7.2). To extend these observations, the proportion of cells in each phase of the cell-cycle before, and at various times after, switching to high extracellular Ca^{++} was determined. Subconfluent keratinocytes were grown in LKGM or HKGM for up to 4 days, with daily replacement of the growth medium, and were then harvested, fixed and the DNA stained with propidium iodide for analysis by flow cytometry (section 5.3).

The DNA histograms obtained from these analyses did not show any major differences between cells maintained in 0.03mM extracellular Ca^{++} (Figure 26 *a*) and cells that had been maintained in 1mM extracellular Ca^{++} for 4 days (Figure 26 *b*). The G_0/G_1 population appears slightly larger in the culture maintained in high extracellular Ca^{++} (*b*), and the S- and G_2/M populations appear to be slightly reduced. Detailed computer analysis of these flow cytometry data (Figure 26 *c*) shows that when keratinocytes were maintained as a subconfluent monolayer in LKGM, 54% were in the G_0/G_1 phase, 24% in S-phase and 22% in G_2/M phase at the time of fixation. These values are in general agreement with the data presented by Wille *et al.,* (1984) - 52% in G_0/G_1, 26% in S-phase and 22% in G_2/M. Switching to HKGM caused only modest alterations in the cell cycle distribution of the culture. One day after switching to HKGM, there was a small increase in the S-phase population, at the expense of the G_0/G_1 and G_2/M phases. After 4 days in 1mM extracellular Ca^{++}, the G_0/G_1 population was slightly larger than in cultures maintained in LKGM, mainly at the expense of the G_2/M phase. These data contradict those of Al-Ani *et al.,* (1988) who showed that the proportion of keratinocytes in the G_1-phase of the cell cycle was decreased in high extracellular Ca^{++}, whilst the proportion of the cells in the S-phase was increased. Their studies were performed in serum-containing medium in the presence of feeder cells

Figure 26

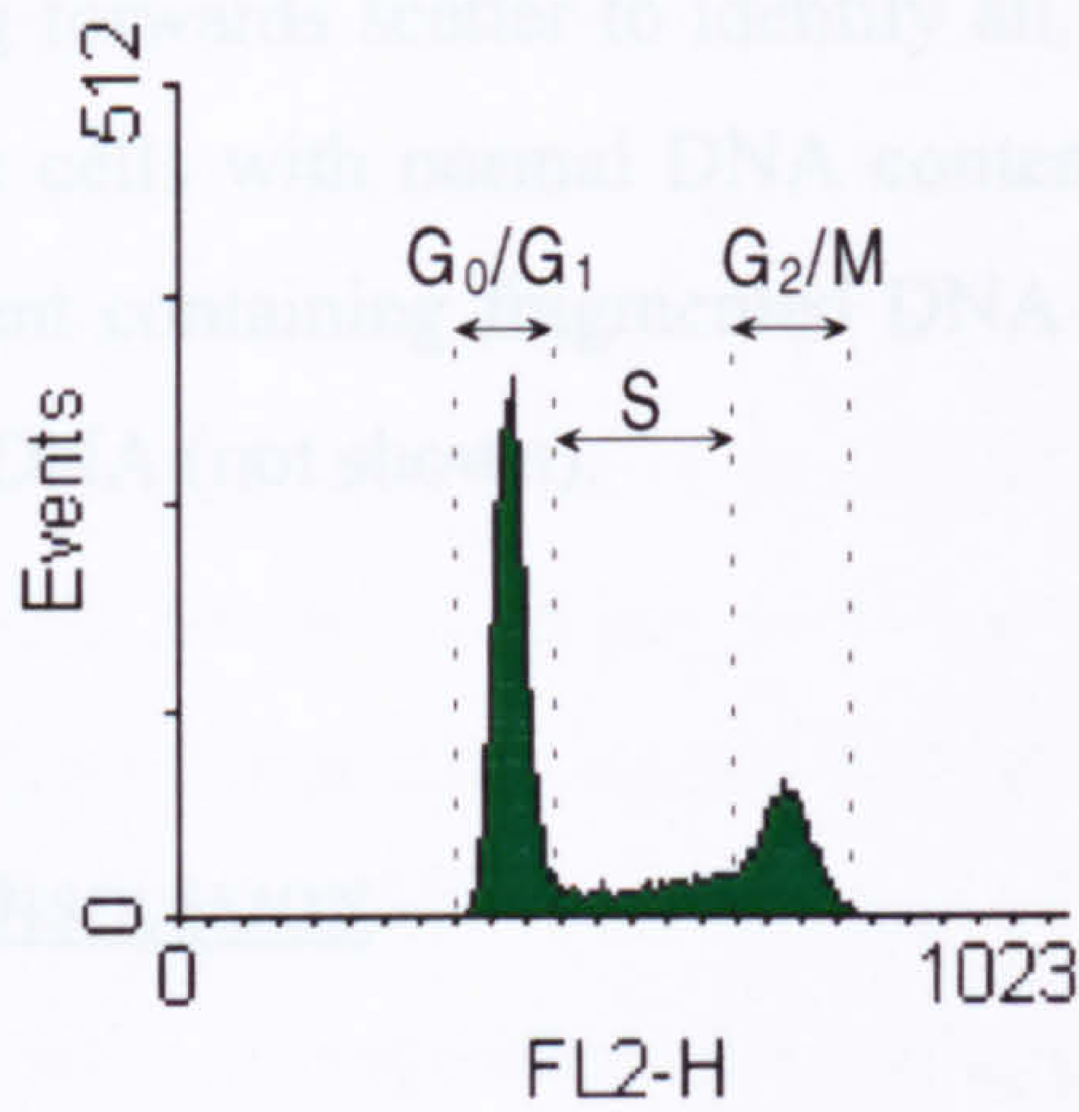
Figure 26

The cell-cycle distribution of keratinocytes grown in low and high extracellular calcium

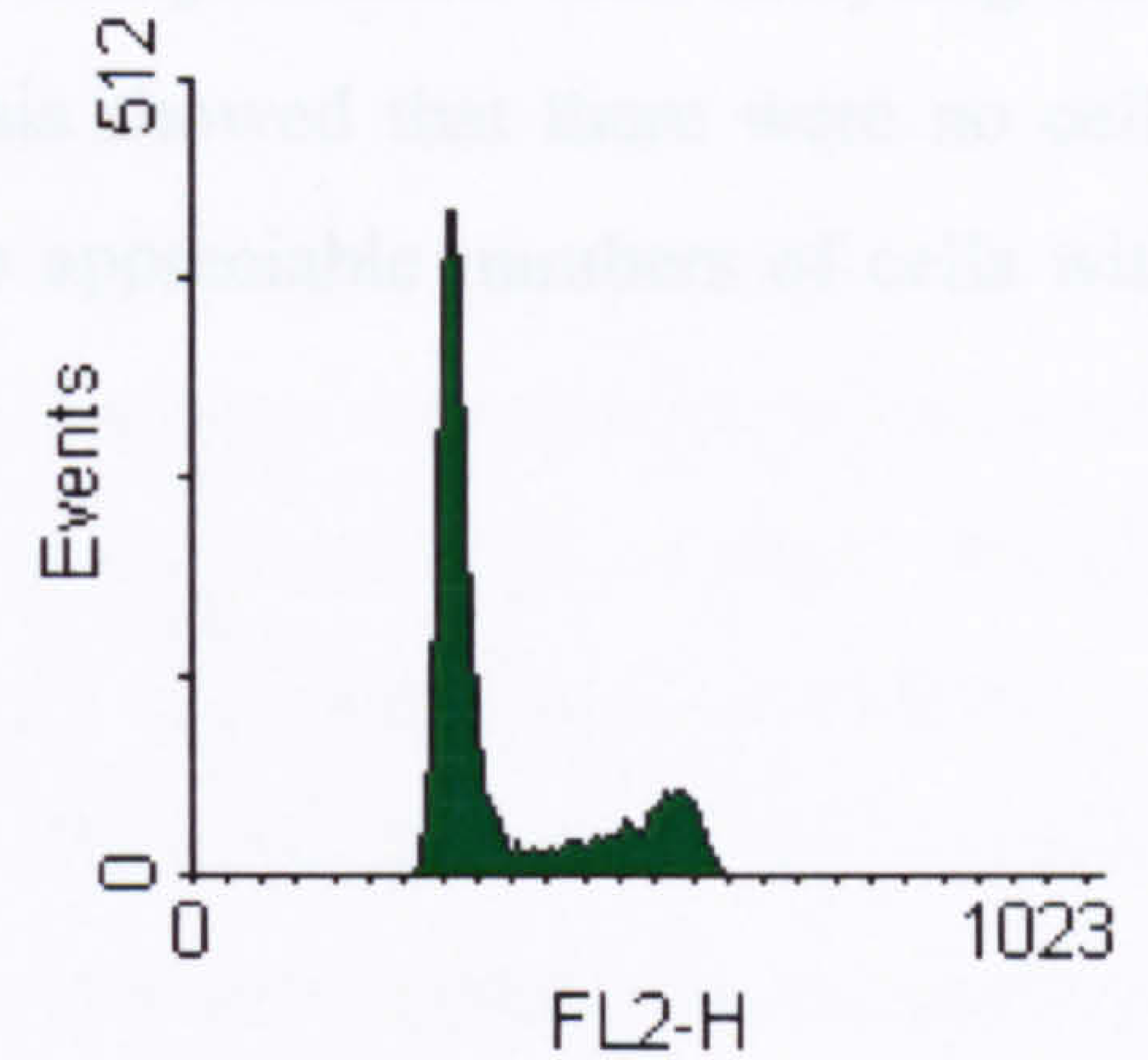
Flow cytometric data depicting the DNA content of cells maintained in 0.03mM or 1mM extracellular Ca^{++} for 4 days (*a* and *b*). These histograms show cell number (*y*-axes) plotted against DNA content (*x*-axes, labelled FL2-H) Computer analysis of DNA content showing the proportion of cells in each phase of the cell cycle at various times after switching to 1mM extracellular Ca^{++} (*c*).

The cell cycle distribution of keratinocytes grown in low and high extracellular calcium

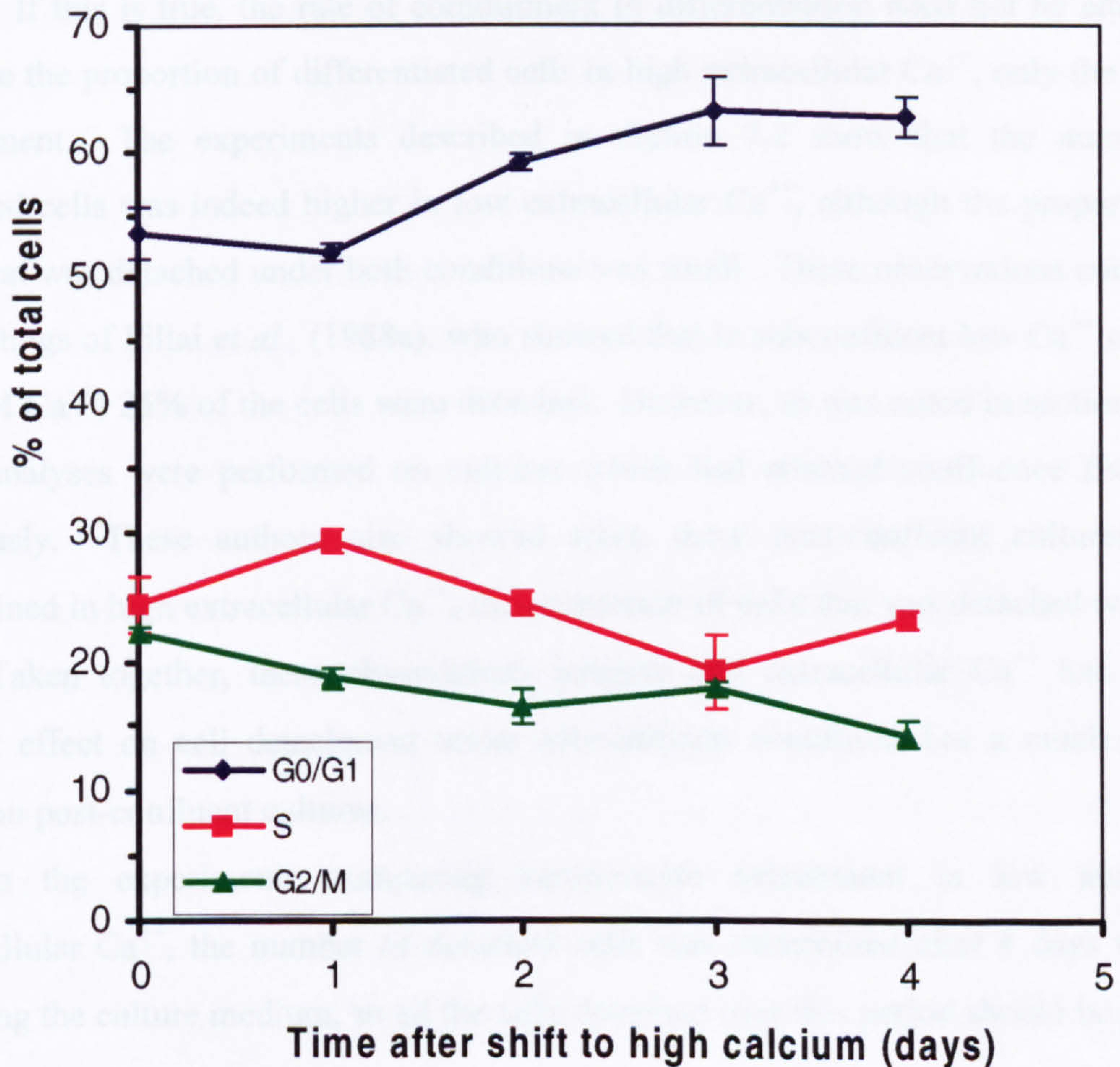
***a.* low extracellular calcium**



***b.* high extracellular calcium, 4 days**



***c.* cell-cycle distribution after switching to high calcium**



and may illustrate an important difference between the serum-containing and serum-free culture systems. Thus, the cell-cycle data presented in this chapter do not support the hypothesis that high extracellular Ca^{++} induces cell-cycle arrest.

To exclude the possibility that any cells with abnormal DNA content were gated out of the analyses by the flow cytometer software, the same samples were also analysed using forwards scatter to identify all the cells in the sample, rather than analysing only those cells with normal DNA content. This analysis showed that there were no cells present containing fragmented DNA nor were there appreciable numbers of cells with >2n DNA (not shown).

7.4 DISCUSSION

7.4.1 Calcium-induced differentiation is not mediated by reduced cell detachment

A possible explanation for the increased proportion of differentiated cells that is seen in high extracellular Ca^{++} is that spontaneously differentiated cells detach in low extracellular Ca^{++} but are retained at higher Ca^{++} concentrations (Drozdoff and Pledger, 1993). If this is true, the rate of commitment to differentiation need not be altered to increase the proportion of differentiated cells in high extracellular Ca^{++}, only the rate of detachment. The experiments described in section 7.2 show that the number of detached cells was indeed higher in low extracellular Ca^{++}, although the proportion of cells that was detached under both conditions was small. These observations contradict the findings of Pillai *et al.,* (1988a), who showed that in subconfluent low Ca^{++} cultures (0.1mM Ca^{++}) 25% of the cells were detached. However, as was noted in section 7.2.2, these analyses were performed on cultures which had attained confluence five days previously. These authors also showed when these post-confluent cultures were maintained in high extracellular Ca^{++}, the proportion of cells that was detached was only 3%. Taken together, these observations indicate that extracellular Ca^{++} has a very modest effect on cell detachment under subconfluent conditions but a much greater effect on post-confluent cultures.

In the experiments comparing keratinocyte detachment in low and high extracellular Ca^{++}, the number of detached cells was determined after 4 days without changing the culture medium, so all the cells detached over this period should be present

in the medium. However, this makes the assumption that all the detached cells remained sufficiently intact that they were counted at the end of the experiment. Normal epithelial cells that are detached from the extracellular matrix undergo apoptosis, a phenomenon known as anoikis (Frisch and Francis, 1994). This is also true for keratinocytes which exhibit degraded DNA and reduced levels of the Bcl-x_L protein when treated in this way (Rodeck *et al.,* 1997). Since it is not clear exactly how long a detached keratinocyte might remain physically intact, the actual number of cells detaching over the duration of the experiment cannot be accurately determined, although no debris was observed in these cultures suggesting that the fragmentation of detached cells did not occur to any great extent. Nonetheless, the experiment showed that the detachment rate was higher in low extracellular Ca^{++} but that it was insufficient to substantially influence the number of attached cells. Thus, the data presented in section 7.2.2 are not consistent with the apparent induction of differentiation by extracellular Ca^{++} being a consequence of the retention of spontaneously differentiated cells that are preferentially shed in low extracellular Ca^{++}.

7.4.2 Calcium-induced differentiation is not mediated by cell-cycle withdrawal

The cell cycle analyses performed on keratinocytes grown in low and high extracellular Ca^{++} (section 7.3) show that high extracellular Ca^{++} did not cause the cells to accumulate in any part of the cell cycle. Some small changes in the cell cycle distribution were apparent, with a small increase in the proportion of cells in the G_1 phase of the cell cycle and small decreases in the S and G_2/M phases over the 4-day duration of the experiment. These data contradict the findings of Al-Ani *et al.,* (1988) who showed a decreased G_1 population after incubation in high extracellular Ca^{++}. The reason for the difference is not clear but may reflect a difference between the serum-containing and serum-free systems. In particular, there may be a different range of growth factors and matrix components available to the keratinocytes in the two systems which could affect their responses to extracellular Ca^{++}. The subtle changes which were observed in the experiments presented in this chapter do not support the hypothesis that the specific arrest of a significant proportion of the cells in a particular phase of the cell cycle is a component of the Ca^{++}-induced differentiation process. However, there are a number of possible scenarios with which these data are consistent.

Firstly, cell-cycle arrest may indeed precede differentiation, but could occur equally in all parts of the cell-cycle. This would not alter the cell-cycle profile of the culture overall but would result in a reduced proliferation rate. However, the cell proliferation data (section 7.2.1) clearly show that this is not the case. A second possibility is that high extracellular Ca^{++} has opposite effects on different cells in the culture. Some cells could growth arrest in different parts of the cell cycle and then differentiate whilst others might proliferate at an increased rate such that overall, the proliferation rate of the culture as a whole was not altered. No data in support of this hypothesis were obtained. The third possibility is that growth arrest does not precede differentiation under these culture conditions and that keratinocytes begin to acquire envelope competence whilst proliferating, only halting proliferation at a much later stage of the differentiation process. This situation would represent a deviation from the normal *in vivo* situation, although under certain circumstances keratinocytes can both proliferate and show features of differentiation simultaneously. For example, suprabasal proliferation is seen in psoriatic epidermis (Weinstein and Van Scott, 1965) in keratinocytes that concurrently express differentiation-specific proteins, although differentiation is not entirely normal (Bernard *et al.,* 1988; Michel *et al.,* 1992). Keratinocyte culture systems such as this and the Rheinwald and Green serum-containing system (section 3.1.1) may represent epidermis undergoing regeneration after wound healing more closely than normal adult epidermis. Under these conditions, enhanced migration occurs and the relationship between proliferation and differentiation may be altered. Indeed, keratinocytes grown in this culture system show some indications that they are hyperproliferative and express keratin K16 (section 1.2.2B, not shown). Nonetheless, these observations show that cell-cycle withdrawal is not an absolute prerequisite for keratinocyte differentiation.

Agents that cause cell-cycle arrest in keratinocytes without cytotoxicity do not necessarily induce differentiation. Transforming growth factor-β (TGFβ) is a potent inducer of cell-cycle arrest in the G_1-phase of cultured keratinocytes (Shipley *et al.,* 1986) but causes a decrease in differentiation (involucrin expression) if used in conjunction with low Ca^{++} medium, demonstrating that cell-cycle arrest does not directly induce differentiation (Matsumoto *et al.,* 1990) under these conditions. Thus, the differentiation program and cell-cycle withdrawal are separable processes in keratinocytes.

7.4.3 High extracellular calcium does not induce apoptosis

Keratinocytes in the spinous and granular layers of adult epidermis show some features of apoptosis, and it has been proposed that keratinocyte differentiation represents a specialised form of apoptosis (Polakowska *et al.,* 1994). This is consistent with the studies performed using suspension culture to induce differentiation, a stimulus which also induces apoptosis in keratinocytes (Rodeck *et al.,* 1997), indicating that the two processes of apoptosis and differentiation may be closely linked. Thus, it is possible that stimuli which induce apoptosis may also induce aspects of differentiation and *vice versa.* However, it should also be noted that there are reports that these two processes are not directly interdependent in keratinocytes (Mitra *et al.*, 1997). Although the occurrence and potential role of apoptosis were not investigated directly as a part of this study, the flow cytometry data indicated that at no stage was fragmented (sub-G_1) DNA present in keratinocyte cultures maintained in low or high extracellular Ca^{++} (Figure 26 *a* and *b*). Since DNA fragmentation is a characteristic feature of apoptosis (Duke *et al.,* 1983; Afanas'ev *et al.,* 1986), these data indicate that high extracellular Ca^{++} does not induce apoptosis under these conditions.

7.5 Summary

The data presented in this chapter indicate that high extracellular Ca^{++} does not indirectly induce keratinocyte differentiation by causing irreversible cell cycle arrest. Furthermore, growth arrest does not appear to be a critical component of the differentiation process under these conditions. The presence of a higher proportion of differentiated cells in the presence of high extracellular Ca^{++} is not a consequence of the retention of spontaneously differentiated cells which would normally detach in low extracellular Ca^{++}, nor is it a consequence of the induction of apoptosis by Ca^{++}. Thus, the formation of cell-cell adhesions may be the response to extracellular Ca^{++} which initiates differentiation and will be further investigated in the next chapter.

CHAPTER 8
CELL-CELL ADHESION AND THE CONTROL OF DIFFERENTIATION

8.1 PURPOSE

In chapter 6, indirect evidence that the formation of intercellular adhesions preceded keratinocyte differentiation was presented. The experiments described in this chapter were designed to test the hypothesis that the formation of cadherin-mediated adherens junctions initiates the *in vitro* keratinocyte differentiation program in response to high extracellular Ca^{++}. To achieve this, two complementary approaches were taken to separate the effects of high extracellular Ca^{++} concentrations and the effects of junction formation. The first of these involved clustering cell-surface E-cadherin in low extracellular Ca^{++} to mimic the formation of adhesions and the second entailed suppressing the formation of cadherin-mediated adhesions in the presence of high extracellular Ca^{++}.

8.2 ANTIBODY-MEDIATED CLUSTERING OF CELL-SURFACE E-CADHERIN

8.2.1 Cell-surface E-cadherin and β_1-integrin can be clustered using antibodies

In order to recreate some aspects of the formation of adherens junctions without elevating the extracellular Ca^{++} concentration, an antibody-mediated technique adapted from Kornberg *et al.* (1991) was used to cluster cell surface E-cadherin in 0.03mM extracellular Ca^{++}. The effect of the formation of these pseudoadhesions on the expression of differentiation-specific proteins by adherent keratinocytes was then examined. The clustering technique involved firstly binding the anti-E-cadherin rat monoclonal antiserum, DECMA-1, to the extracellular portion of cell-surface E-cadherin. This stage was performed on chilled cells to prevent the cadherin-antibody complex from being internalised during the procedure. After washing away the unbound DECMA-1, goat anti-rat antiserum was added to cluster the cadherin-DECMA-1 complexes and the cells were returned to a 37°C incubator. A cell treated in this way is depicted schematically in Figure 27 *a*. Confirmation that E-cadherin

clustering had been achieved was obtained by staining the cells with a mouse anti-E-cadherin antiserum which recognises the intracellular domain of E-cadherin and then visualising this antibody by confocal immunofluorescence microscopy (Figure 27 *b*). As is apparent in the image, the clustering technique resulted in the aggregation of E-cadherin into discrete patches on the cell surface which were often circular in shape, a staining pattern which contrasts with that seen in untreated cells (Figure 16 *a*) Not all the aggregates present on the surface of the cells are visible as some are outwith the plane of focus of this confocal image. Thus, E-cadherin can be clustered on the surface of adherent keratinocytes in low extracellular Ca^{++}.

The data presented in chapter 6 indicated that integrin-mediated cell-cell contacts were also formed in response to high extracellular Ca^{++}. Therefore, the β_1-integrin subunit was clustered on the cell surface in low extracellular Ca^{++} in the same way as described for E-cadherin. Figure 27 *c* shows a small group of cells stained for the β_1-integrin subunit after the clustering process had been performed. A number of discrete clusters of this integrin were visible on the cell surface, similar to those shown in Kornberg *et al.* (1991), in contrast to the diffuse staining pattern seen on untreated cells (Figure 22 *a*). Thus, β_1-integrin can be clustered on the surface of adherent keratinocytes in low extracellular Ca^{++}. This integrin clustering provided a valuable control for the E-cadherin clustering as both of the first antisera are rat monoclonal antibodies of the same immunoglobulin subclass, permitting the use of the same secondary antibody for both clustering procedures.

For the clustering experiments, control cells were treated with the different antibodies used for clustering individually, i.e. DECMA-1 with no second antibody, or goat anti-rat alone. These control treatments did not result in the aggreagation of E-cadherin or β_1-integrin into patches on the cell surface (not shown), indicating that the staining patterns shown in Figure 27 *b* and *c* were specific to cells treated to cluster E-cadherin or β_1-integrin.

Figure 27

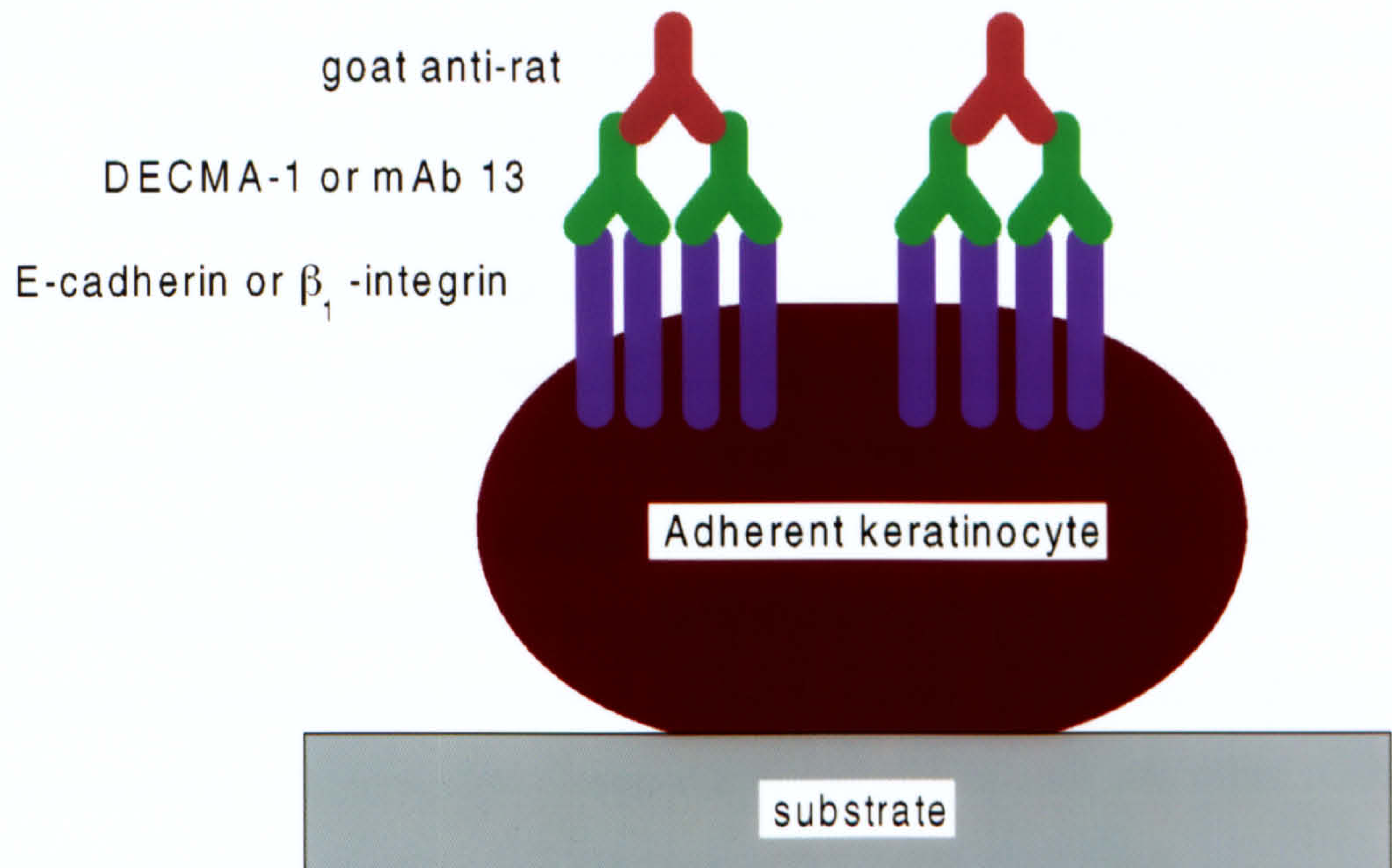
Figure 27

Antibody-mediated clustering of cell-surface molecules

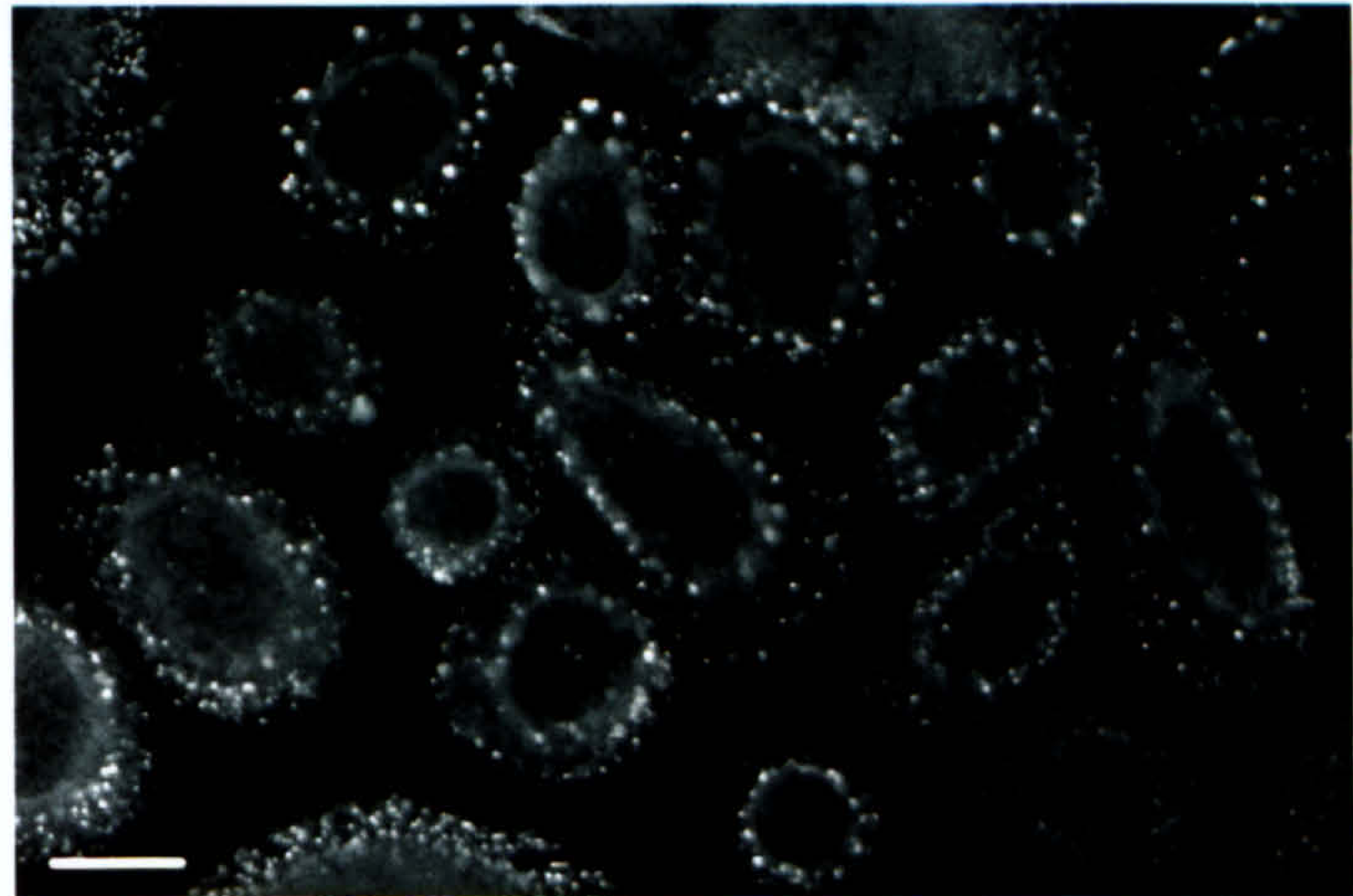
This figure shows a schematic representation summarising the technique used to cluster cell-surface E-cadherin or β_1-integrin (*a*) and confocal immunofluorescence micrographs of cells treated to cluster E-cadherin (*b*) and β_1-integrin (*c*) in this way. The antisera used for staining these cells were mouse anti-E-cadherin (clone #36) and rat anti-β_1-integrin (mAb 13) diluted 1:250 and 1:100 respectively and visualised with FITC anti-mouse or FITC anti-rat. The scale bars represent 25μm.

Antibody-mediated clustering of cell-surface molecules

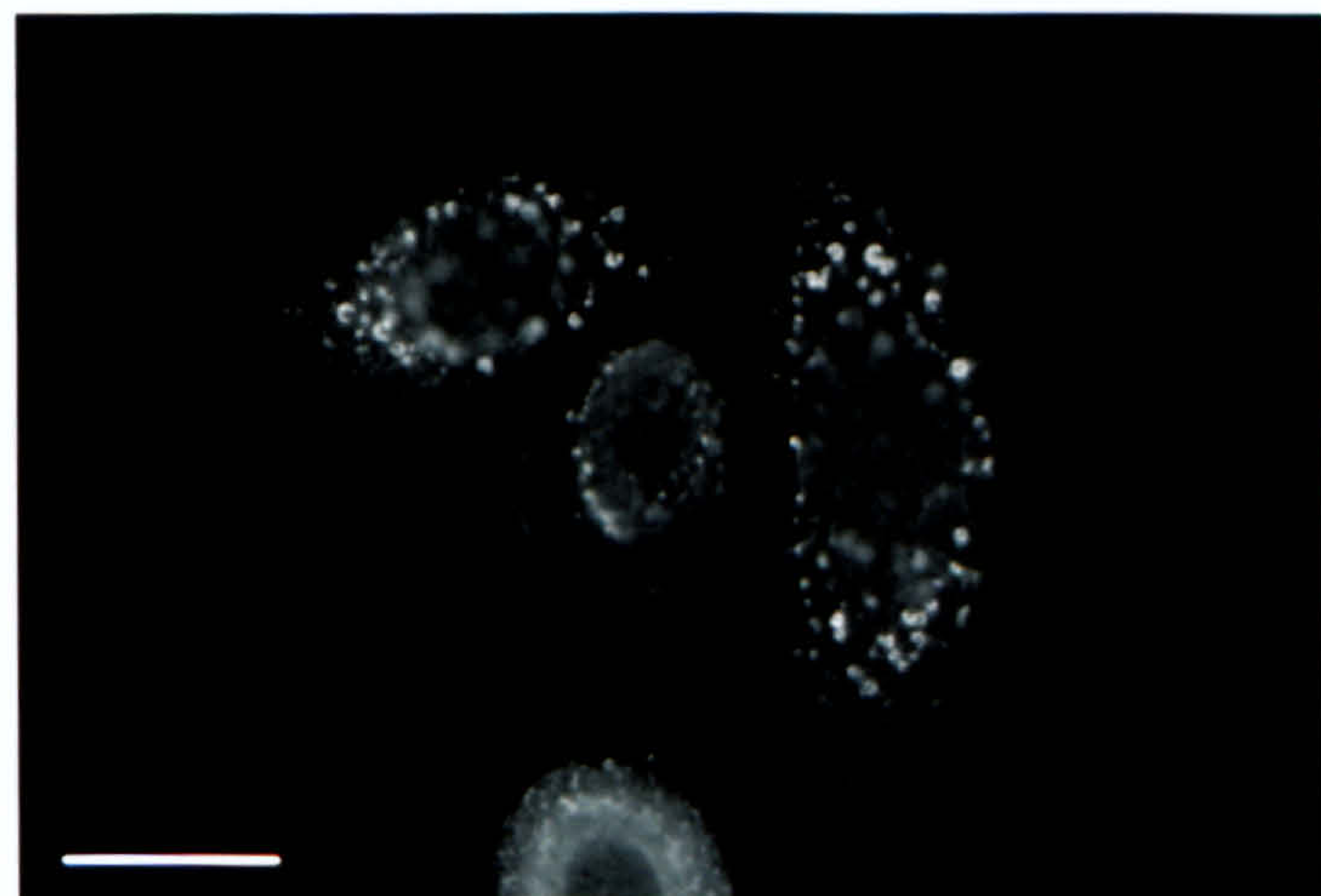
a. **schematic representation of the clustering process**



b. **clustered E-cadherin**



c. **clustered β_1 -integrin**



8.2.2 E-cadherin clustering induces involucrin and TGk expression

In order to determine the effects of E-cadherin and β_1-integrin clustering on keratinocyte differentiation, cells growing on chamber slides were treated to cluster either E-cadherin or β_1-integrin as described above, washed to remove excess anti-rat antiserum and then incubated in antibody-free LKGM for 4 days. During this time, the cells did not reach confluence. At the end of this 4 day period, the cells were fixed and stained for involucrin or TGk using the immunoperoxidase method (section 5.12).

After staining for the expression of involucrin and TGk, numerous random fields were photographed using a phase-contrast microscope and these photographs were then used to calculate the proportion of cells expressing involucrin or TGk. These data are presented in Figure 28 with chart *a* summarising the data for involucrin and chart *b* summarising the data for transglutaminase. In both charts, only the cells represented by the first column (shown in red) have been treated to cluster E-cadherin, with the blue columns representing cells treated to cluster the β_1-integrin subunit and other controls. The precise combinations of antibodies used are shown below each chart.

When no antibodies were added to the culture medium, very few cells were stained for involucrin or transglutaminase 4 days later (Figures 28 *a* and *b*). Similarly, treating the cells with DECMA-1 or mAb 13 alone had no effect on the proportion of cells which stained strongly for involucrin or TGk under these subconfluent growth conditions. The $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are important mediators of cell-matrix interactions in keratinocytes (Carter *et al.,* 1990a) and yet treating the cells with mAb 13, which blocks β_1-mediated adhesion (Akiyama *et al.,* 1989) did not induce cell detachment (not shown). In addition, treatment with goat anti-rat antiserum alone had no effect on the proportion of cells expressing either involucrin or TGk. When cells were treated to cluster the β_1-integrin subunit, no increased staining for involucrin or transglutaminase was observed (data not shown for transglutaminase).

Clustering E-cadherin increased the proportion of cells which stained for involucrin and TGk. For involucrin, E-cadherin clustering resulted in almost 60% of the cells staining for this differentiation-specific protein, corresponding to a 3.5-fold increase over the control cells. The proportion of cells strongly expressing TGk in response to E-cadherin clustering was lower at 35%, but this corresponded to a 7-fold increase over the control level, reflecting the lower basal levels of TGk expression in the

control cells. Thus, clustering cell-surface E-cadherin in adherent keratinocytes maintained in low extracellular Ca^{++} increased the proportion of cells expressing involucrin and transglutaminase whereas clustering β_1-integrin did not.

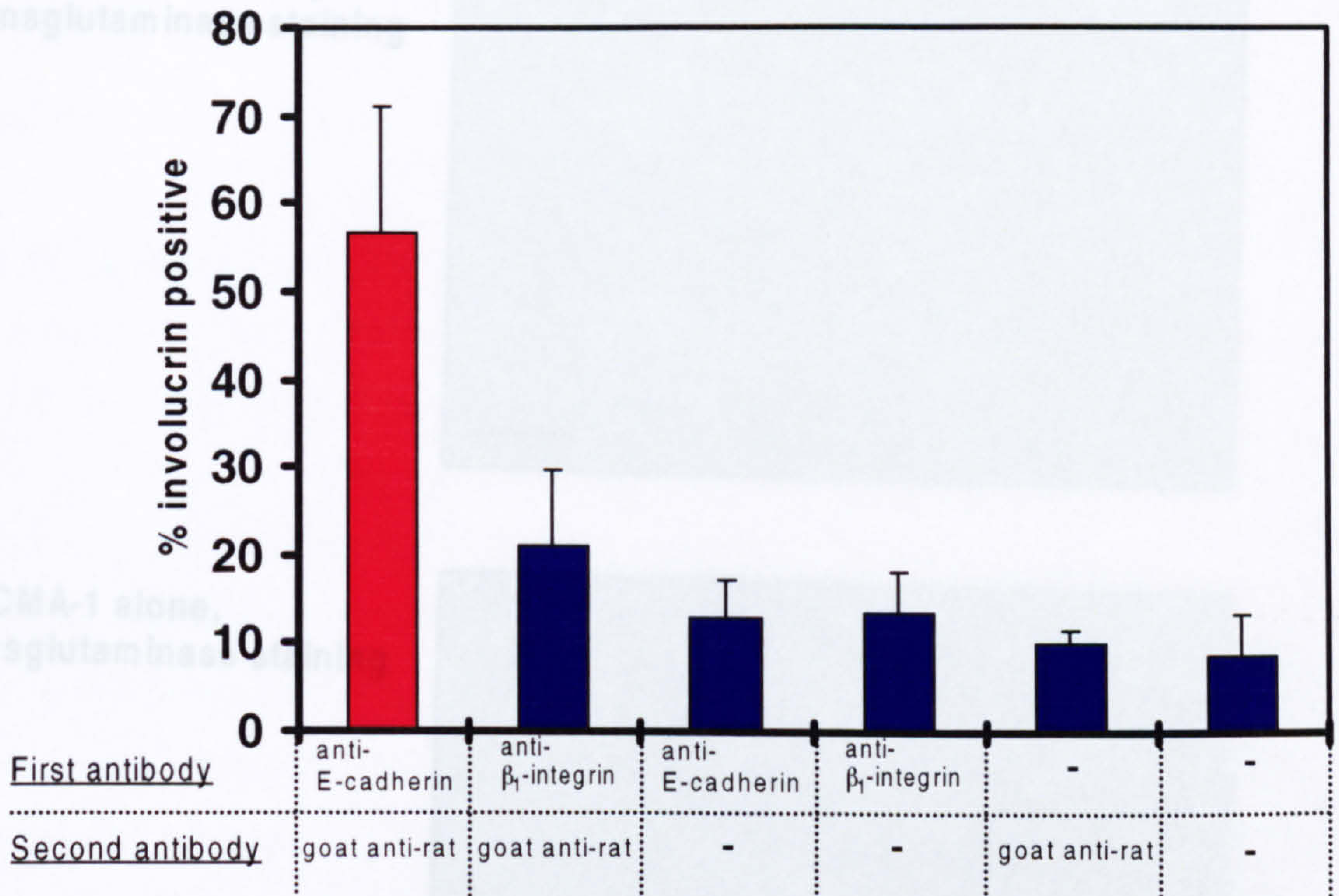
Figure 28

Figure 28
Involucrin and transglutaminase induction by E-cadherin clustering

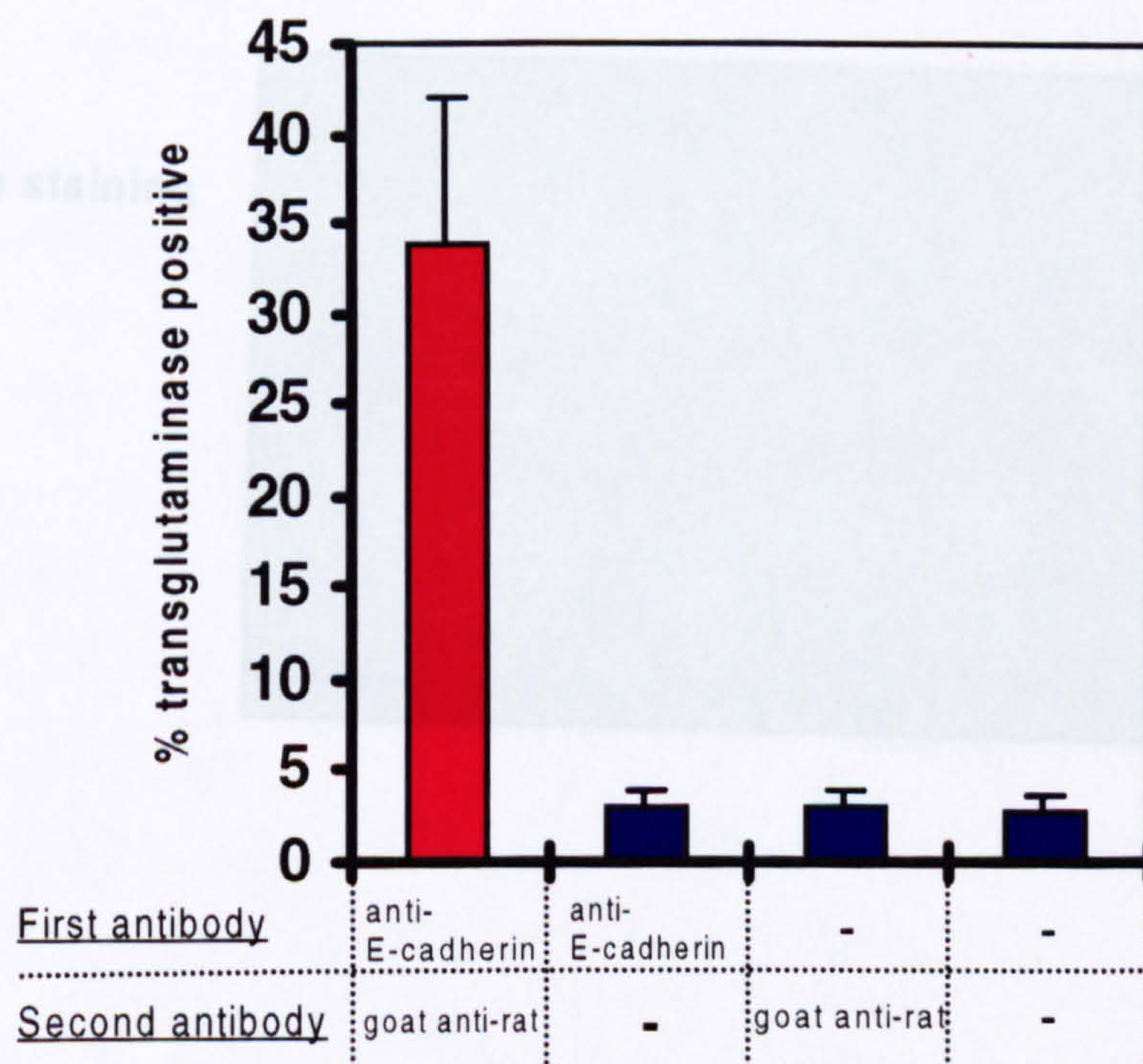
Summaries of the quantification of the proportion of cells strongly staining for involucrin (*a*) or TGk (*b*) after antibody-mediated clustering of E-cadherin (red column) or control treatments (blue columns). For each chart, between 1×10^4 and 2×10^4 cells were counted on photographs which had been randomly relabelled by an independent observer so that the counting was performed blind. Each column represents the mean of four or more samples, + SEM.
Phase-contrast photomicrographs showing adherent keratinocytes stained for transglutaminase 4 days after antibody-mediated clustering of E-cadherin (*c*), or treatment with first antibody alone (*d*) or after no antibody treatments (*e*). The antiserum used was anti-transglutaminase, mAb B. C1 diluted 1:1000. Scale bars represent 200μm.

Involucrin and transglutaminase induction by E-cadherin clustering

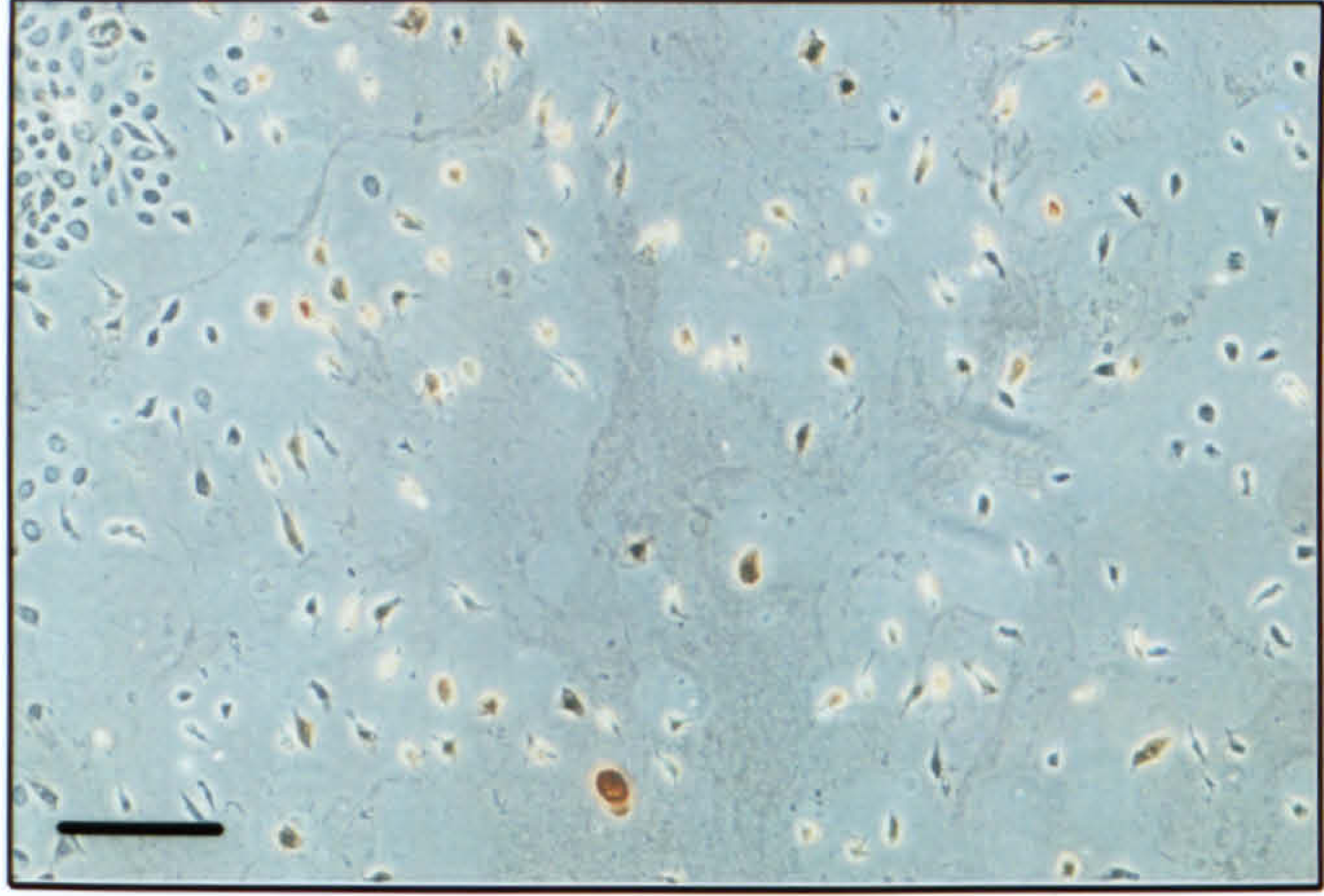
a. Involucrin



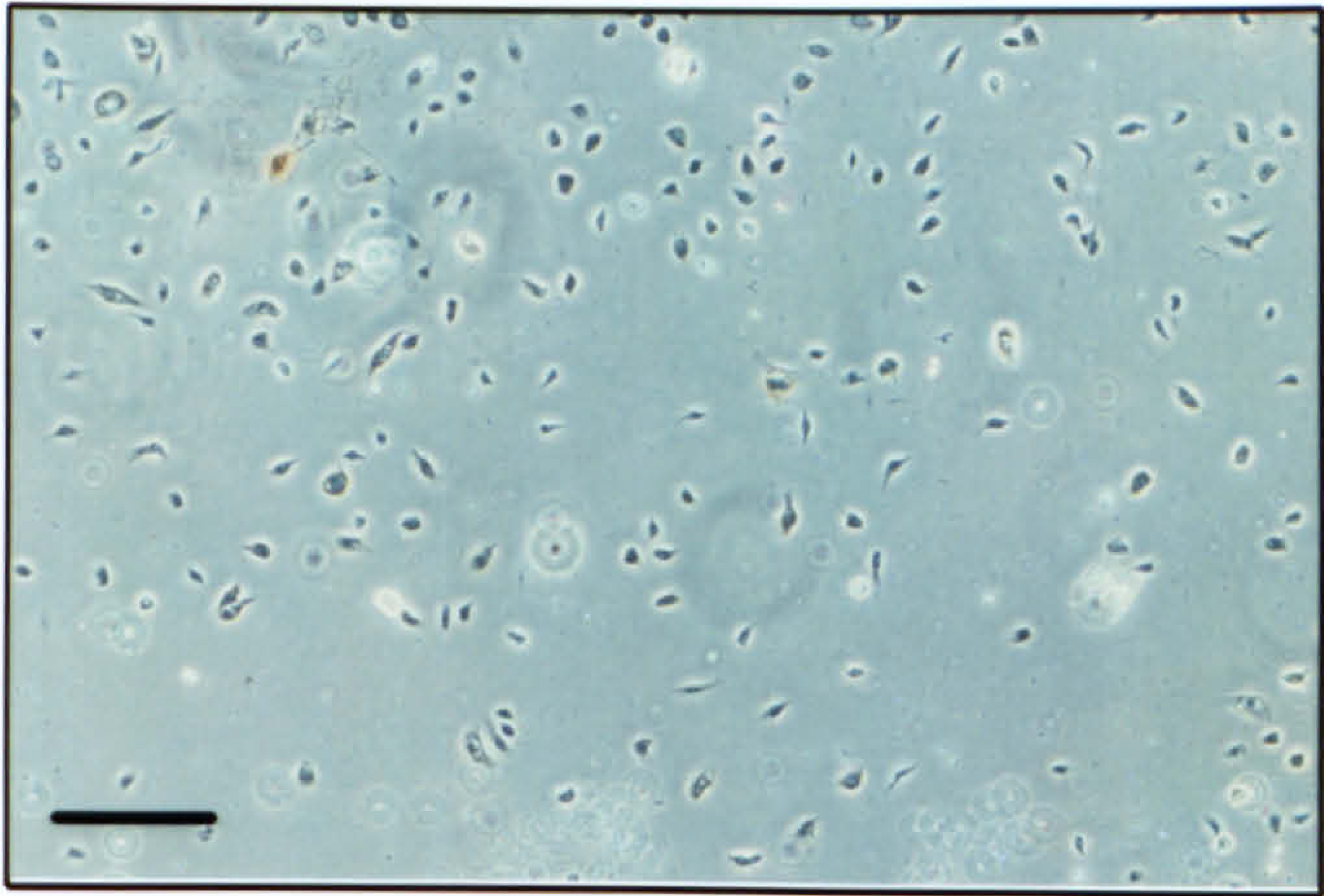
b. Transglutaminase



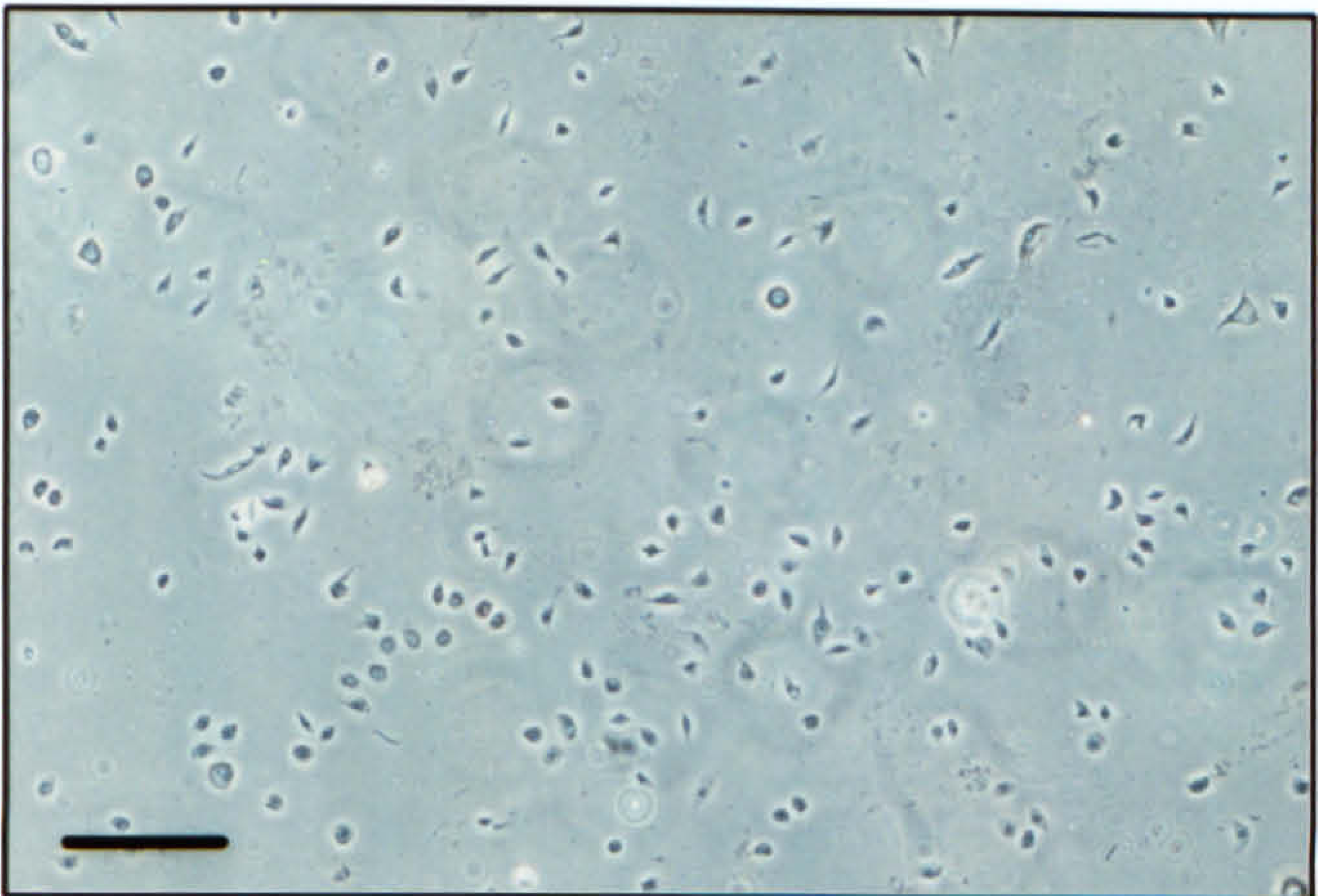
c. E-cadherin clustering,
transglutaminase staining



d. DECMA-1 alone,
transglutaminase staining



e. no clustering,
transglutaminase staining



8.3 Inhibition of adherens junction formation using synthetic peptides

A complementary approach to the cadherin clustering experiments was to suppress the formation of cadherin-mediated adherens junctions in the presence of high extracellular Ca^{++}. These experiments were designed to test the hypothesis that it is the formation of the adherens junctions in response to high extracellular Ca^{++} that initiates differentiation, rather than some other cellular response to this stimulus.

8.3.1 The cadherin-blocking synthetic peptides

The method adopted to interfere with cadherin-mediated cell-cell adhesion was to treat the keratinocytes with synthetic peptides designed to compete with the initial interactions between cadherins. The blocking decapeptide used for these studies (LRAHAVDVNG-amide) contains the conserved HAV sequence (section 2.2.2), and is identical to the peptide described by Blaschuk *et al.,* (1990) which interferes with E-cadherin and N-cadherin mediated adhesion. The peptide is homologous to one of the β-strands (the F-strand) of the EC-1 domain of avian N-cadherin (Figure 29), believed to be the site where the initial recognition between cadherins on opposing cells takes place. The amino acids shown in parentheses in Figure 29 are the corresponding residues in human E-cadherin, of which 60% are identical to those of avian N-cadherin. A control peptide (VIPPINLPEN-amide), identical to the control peptide used by Blaschuk *et al.,* corresponds to a sequence of avian N-cadherin that is not believed to participate in the adhesion process.

8.3.2 Cadherin-blocking peptides suppress calcium-induced differentiation

To investigate the effects of preventing the formation of cadherin-mediated adherens junctions in high extracellular Ca^{++} on the expression of differentiation-specific proteins, the following approach was taken. Adherent keratinocytes maintained in LKGM on chamber slides were treated with blocking or control peptide at a concentration of 0.2mg/ml for 30 minutes and were then either switched to HKGM or maintained in LKGM. The cells were then maintained in LKGM/HKGM in the presence of control or blocking peptide (replaced daily) for 4 days and were then fixed and stained for transglutaminase using the immunoperoxidase method.

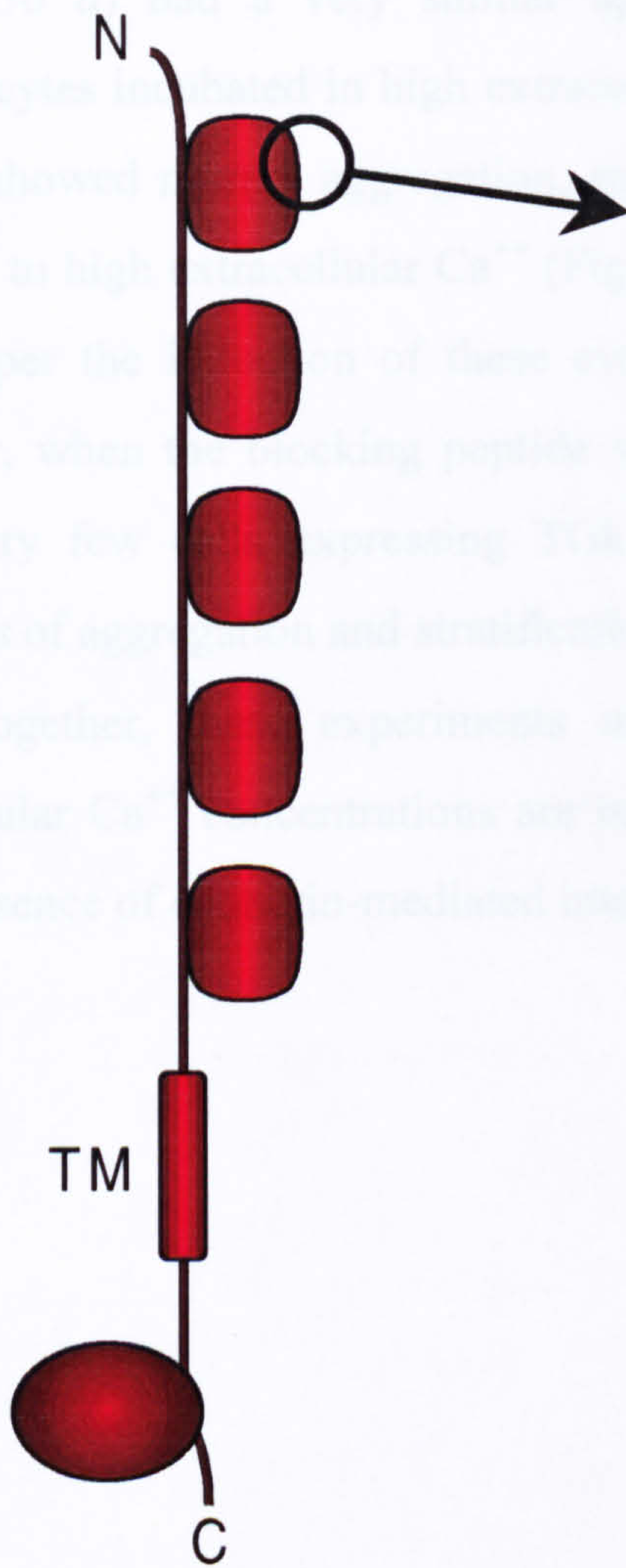
Figure 29

Figure 29
Derivation of the cadherin-blocking peptide

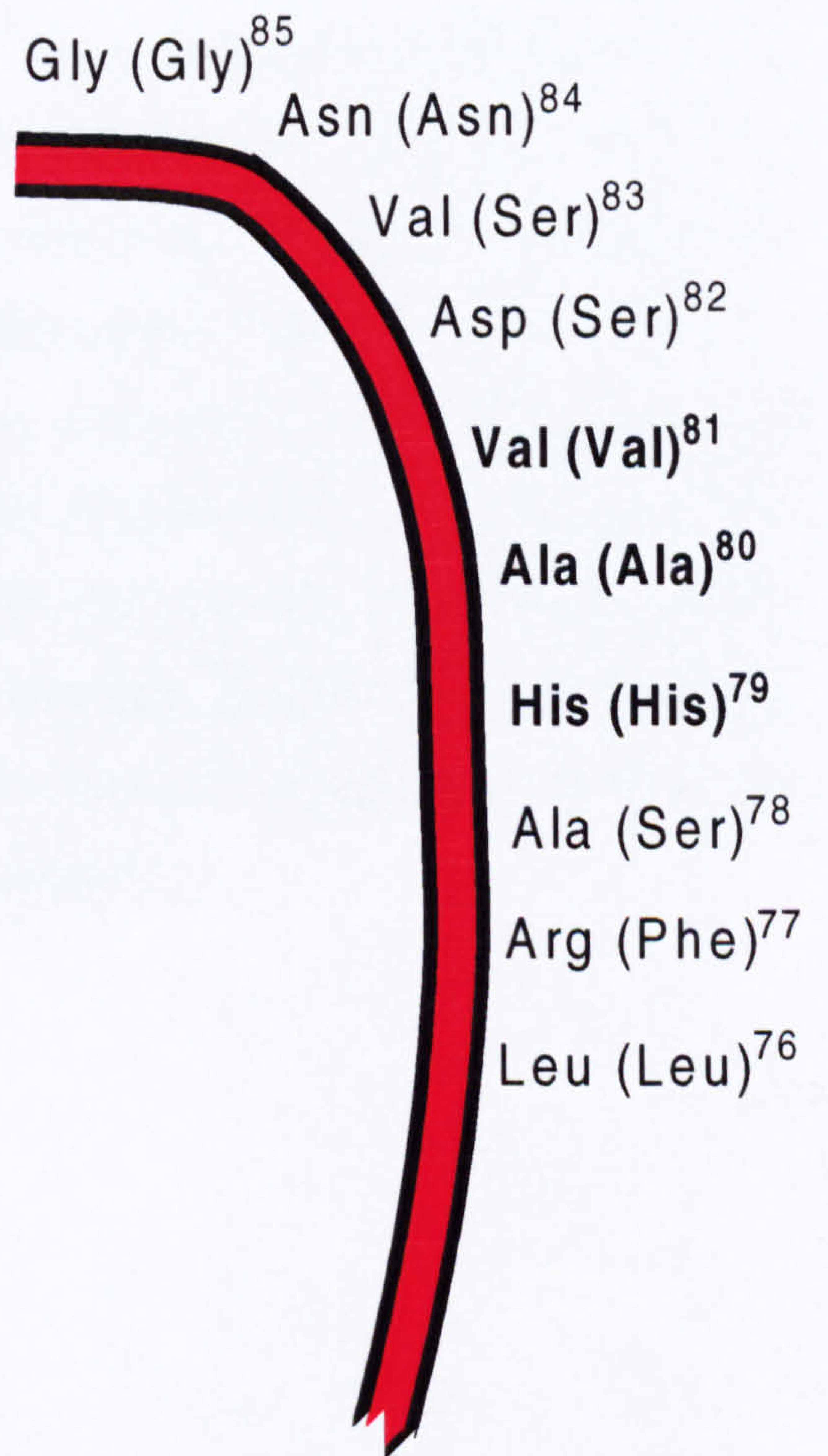
This figure shows a representation of N-cadherin showing the localisation of β-strand F (βF) and the portion of this which was synthesised to produce a peptide capable of blocking cadherin function (N denotes the amino-terminus, TM denotes the transmembrane domain and C denotes the carboxy terminus). The three conserved residues ($H^{79}A^{80}V^{81}$) that are believed to comprise the cadherin recognition sequence are indicated in bold font. In murine E-cadherin, which is highly homologous to avian N-cadherin in this region, it is predicted that His^{79} and Val^{81} are solvent-exposed (Overduin *et al.,* 1995). The amino acid residues indicated are those present in avian N-cadherin and the synthetic peptide whilst those given in parentheses are the corresponding residues present in human E-cadherin.

Derivation of the cadherin-blocking peptide

N-cadherin



βF-strand



When treating keratinocytes with the blocking peptide, it was found that the peptide was insufficient to disrupt pre-existing cell-cell adhesions (not shown) which form in 0.03mM extracellular Ca^{++} if the cells are allowed to reach high density (section 8.4.2). Thus, to enable the peptide to successfully prevent cell-cell adhesion, the cells were maintained at low density prior to the addition of the peptide.

The effects of blocking the formation of cell-cell adhesions on transglutaminase expression are shown in Figure 30. Keratinocytes that were maintained in low extracellular Ca^{++} in the presence of control peptide (Figure 30 *b*) or blocking peptide (Figure 30 *a*) had a very similar appearance and expressed low levels of TGk. Keratinocytes incubated in high extracellular Ca^{++} for 4 days in the presence of control peptide showed normal aggregation, stratification and increased expression of TGk in response to high extracellular Ca^{++} (Figure 30 *d*), indicating that the control peptide did not hamper the induction of these events by high extracellular Ca^{++} concentrations. However, when the blocking peptide was used in conjunction with high extracellular Ca^{++}, very few cells expressing TGk were seen (Figure 30 *c*). Furthermore, the processes of aggregation and stratification were also suppressed by the blocking peptide. Taken together, these experiments using the blocking peptide indicate that high extracellular Ca^{++} concentrations are insufficient to induce keratinocyte differentiation in the absence of cadherin-mediated intercellular adhesion.

Figure 30

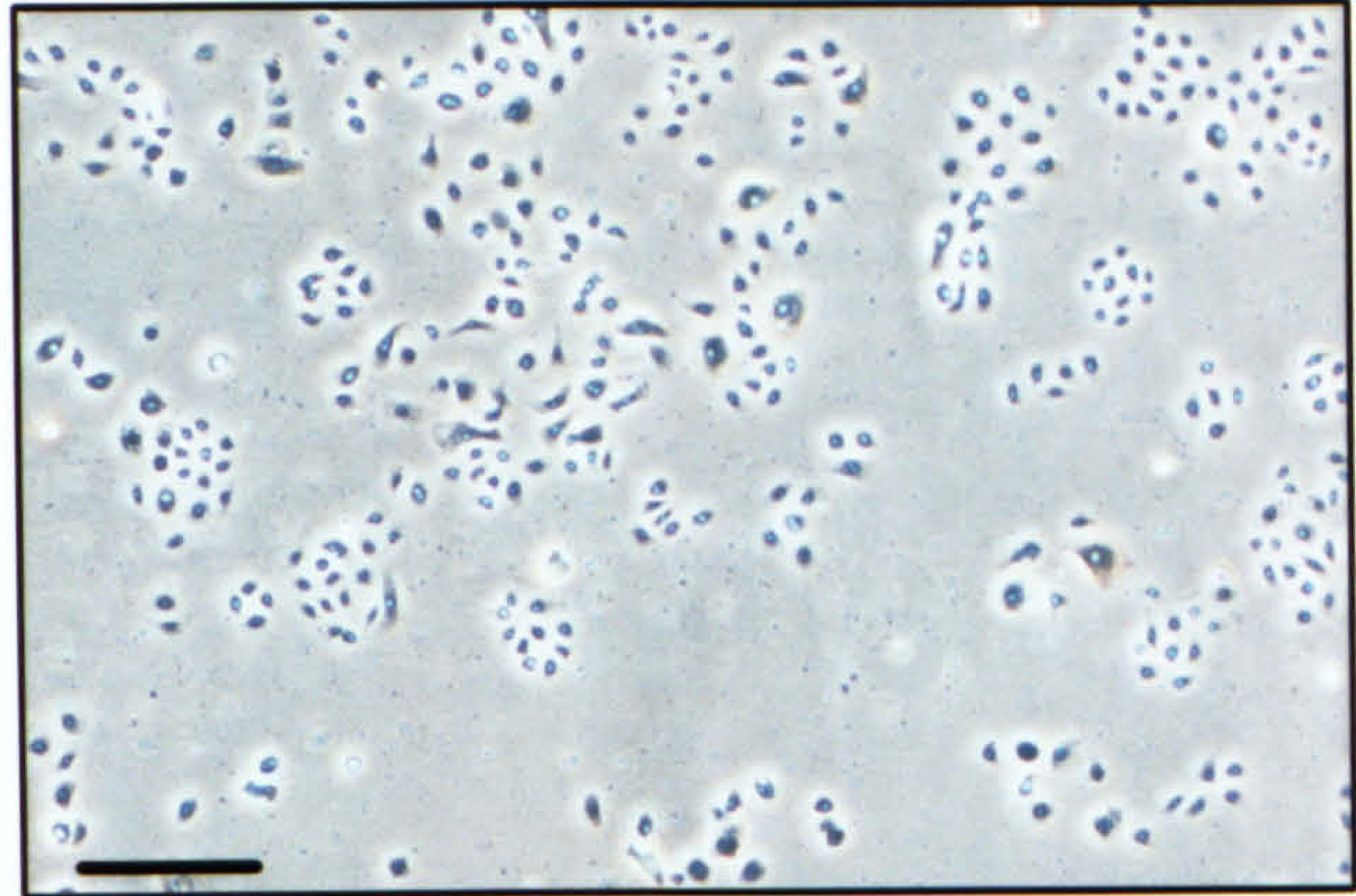
Figure 30

Transglutaminase expression in cells treated with cadherin-blocking peptides

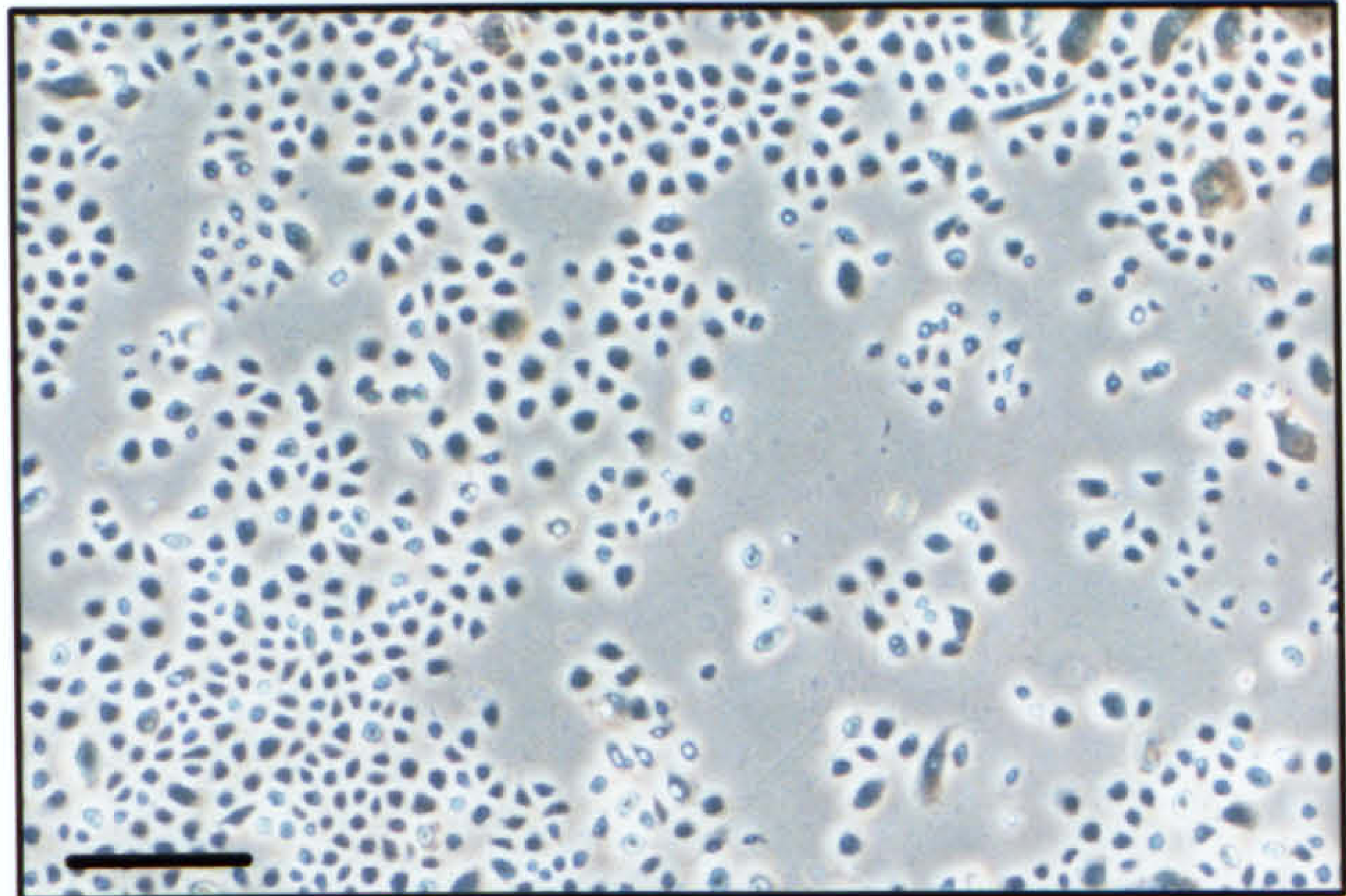
Phase-contrast photomicrographs of adherent keratinocytes stained for transglutaminase using the immunoperoxidase method after maintaining in low extracellular Ca^{++} (*a* and *b*) or in high extracellular Ca^{++} for a period of 4 days (*c* and *d*). The cells shown in panels *a* and *c* were treated with cadherin-blocking peptide for the duration of the experiment whilst the cells shown in panels *b* and *d* were treated with control peptide. The peptides were used at a concentration of 0.2mg/ml and were added to the cells 30 minutes before switching to 1mM extracellular Ca^{++} where appropriate. The culture medium and peptide were replaced daily. The antiserum used was mAb B. C1 diluted 1:1000. Scale bars represent 200μm.

Transglutaminase expression in cells treated with cadherin blocking peptide

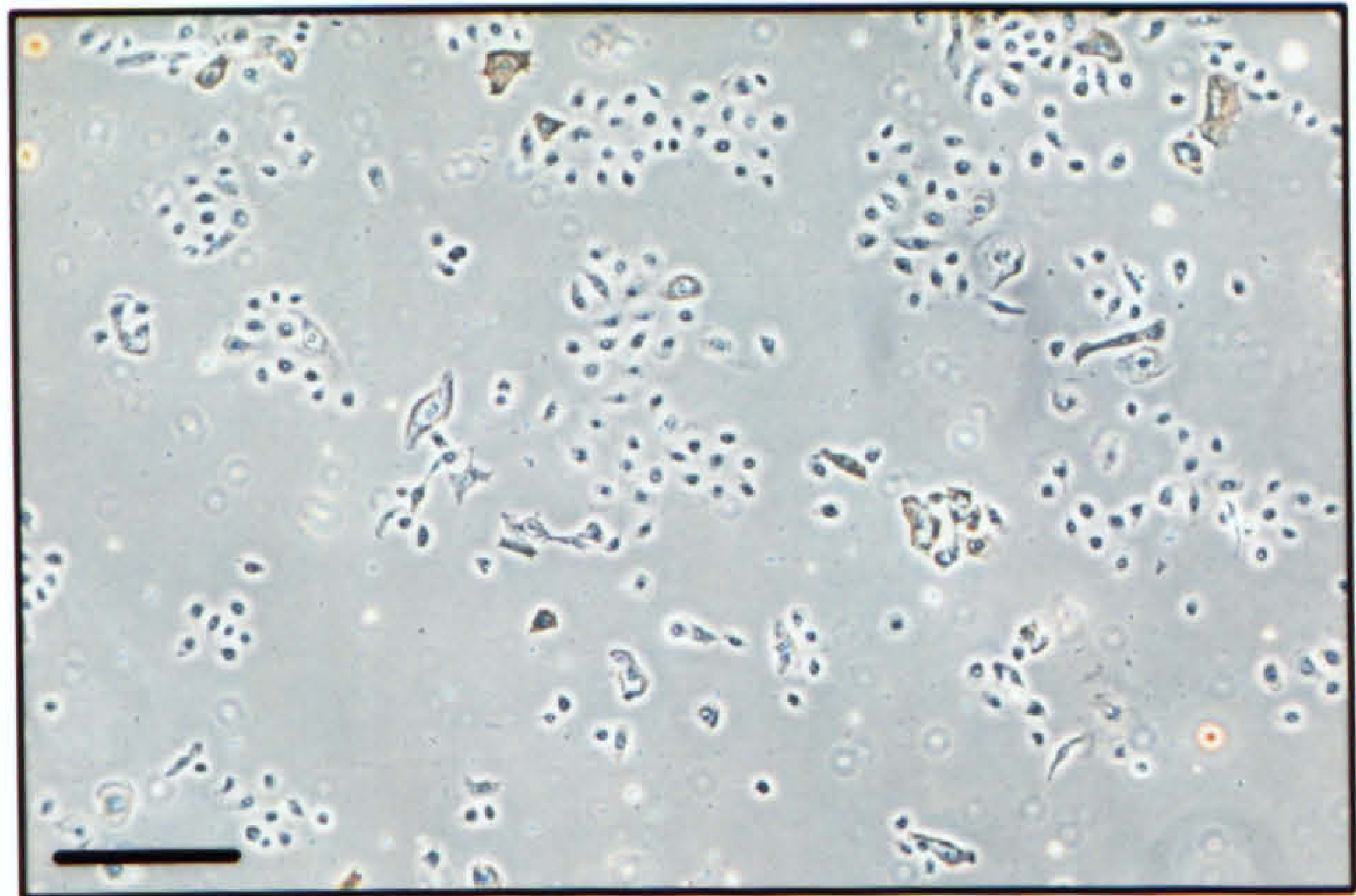
a. low extracellular calcium, blocking peptide



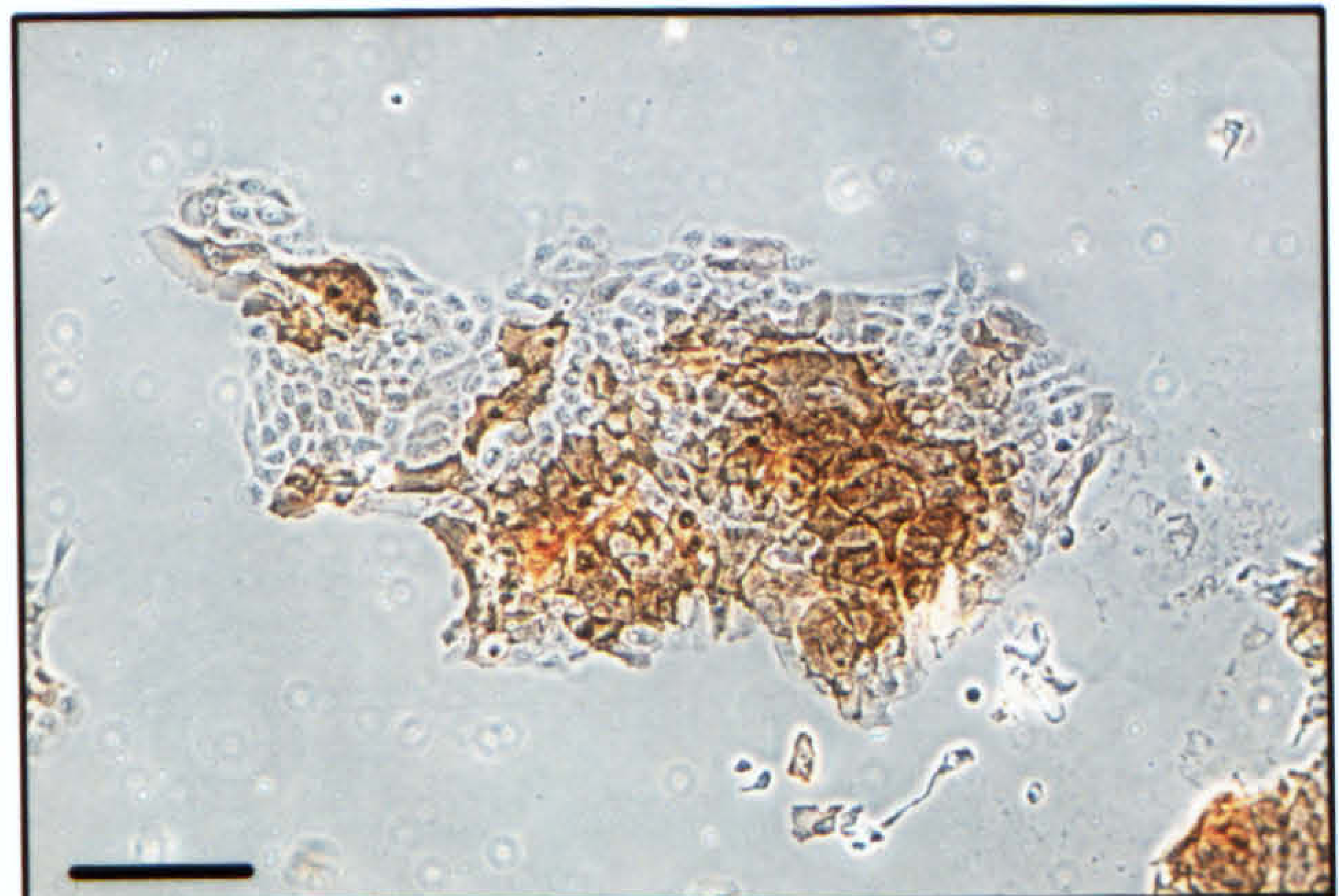
b. low extracellular calcium, control peptide



c. high extracellular calcium, blocking peptide



d. high extracellular calcium, control peptide



8.4 HIGH CELL DENSITY AND KERATINOCYTE DIFFERENTIATION

8.4.1 High cell density induces increased involucrin expression

The experiments described in sections 8.2 and 8.3 were performed using cells that were never allowed to reach confluence, as previous studies have indicated that prolonged growth at high density can induce differentiation, even in low extracellular Ca^{++} (Poumay and Pittelkow, 1995; Pillai *et al.*, 1988a). Since low extracellular Ca^{++} conditions are thought to suppress cadherin-mediated cell-cell adhesion, this observation indicates that differentiation induced by high cell density may be initiated in a cadherin-independent manner. To investigate the relationship between cell-cell adhesion and high density-induced differentiation, involucrin expression and subcellular cadherin localisation were determined in post-confluent keratinocytes maintained in LKGM. Keratinocyte cultures were allowed to reach confluence in low extracellular Ca^{++} and were maintained for a further 5 days with daily medium replacement. These cultures were propagated in amino acid-enriched LKGM (section 4.11.1) to minimise the possibility of nutrient depletion. Protein extracts were prepared from post-confluent cultures at daily intervals and were immunoblotted for involucrin (Figure 31). Similar experiments were performed on cells grown on chamber slides to allow involucrin immunoperoxidase staining to be carried out (Figure 32).

Immunoblotting showed that maintaining keratinocytes at high density in low extracellular Ca^{++} (Figure 31 *a*) induced the expression of involucrin to a similar extent as treatment with high extracellular Ca^{++} (Figure 31 *b*). The kinetics of involucrin induction by these two stimuli are difficult to compare as keratinocytes grown under these conditions did not attain confluence simultaneously, with some areas of cells reaching confluence before others. However, these data show that prolonged culture at high density provides a stimulus that is able to induce involucrin expression similar to 1mM extracellular Ca^{++}.

Occasional spontaneously differentiated cells which stained for involucrin were present in subconfluent cultures maintained in LKGM (Figure 32 *a*, arrow). However, five days after confluence was attained, a much larger proportion of the cells stained strongly for involucrin, confirming the data obtained by immunoblotting (Figure 32 *b*). The cells which stained for involucrin were generally larger than their non-differentiated counterparts, whether differentiation was spontaneous or induced by high culture

density. No stratification (section 6.4) was seen in cultures where the keratinocytes were induced to differentiate by prolonged culture at high density although occasional weakly attached rounded cells were sometimes visible on the upper surface of the monolayer (Figure 32 *b*, arrow). These are likely to represent cells physically forced away from the substrate by their neighbours. Thus, prolonged growth at high density is sufficient to induce involucrin expression in a large proportion of the cells, consistent with a role for cell-cell contact in the induction of differentiation.

8.4.2 High cell density induces the formation of adherens junctions in low extracellular calcium

To investigate the effects of enforced cell-cell contact induced by high cell density on intercellular adhesion, post-confluent keratinocytes were stained for E-cadherin as described in section 6.5.1. The cells were maintained in amino-acid enriched LKGM (section 4.11.1) for 5 days after confluence was attained before staining. Even though these cells were maintained in low extracellular Ca^{++} throughout the experiment, extensive staining for E-cadherin was seen at cell-cell contact areas (Figure 33) although the staining intensity was weaker than that induced by high extracellular Ca^{++} (Figure 16). Thus, high culture density in low extracellular Ca^{++} is sufficient to induce the relocalisation of some E-cadherin to sites of cell-cell contact consistent with a role for E-cadherin in mediating high density-induced differentiation.

Figure 31

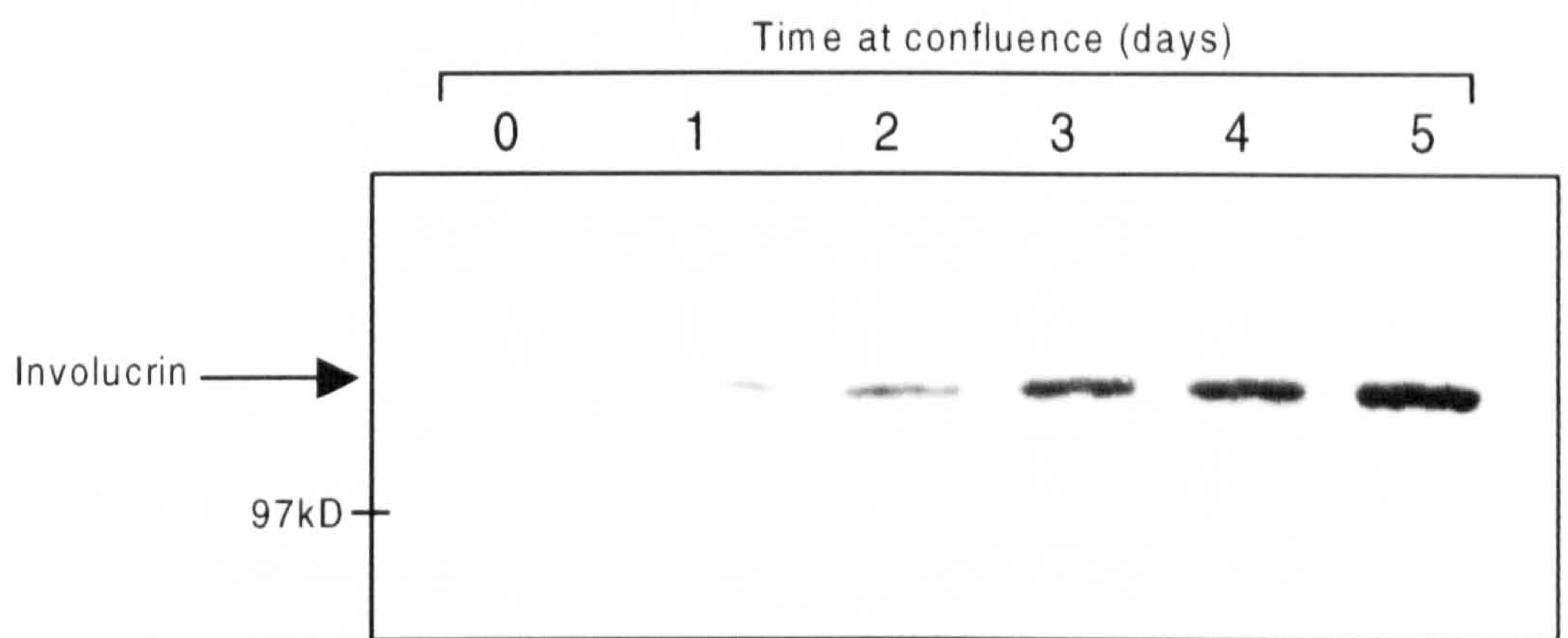
Figure 31

Prolonged high cell density induces involucrin expression

Immunoblots for involucrin prepared using proteins extracted using CSK buffer (section 4.11.2) from keratinocytes grown to confluence and maintained post-confluence (*a*) for the times indicated or treated with high extracellular Ca^{++} (*b*). The antiserum used was anti-involucrin rabbit polyclonal, diluted 1:500.

Prolonged high cell density induces involucrin expression

***a.* high cell density**



***b.* high extracellular calcium**

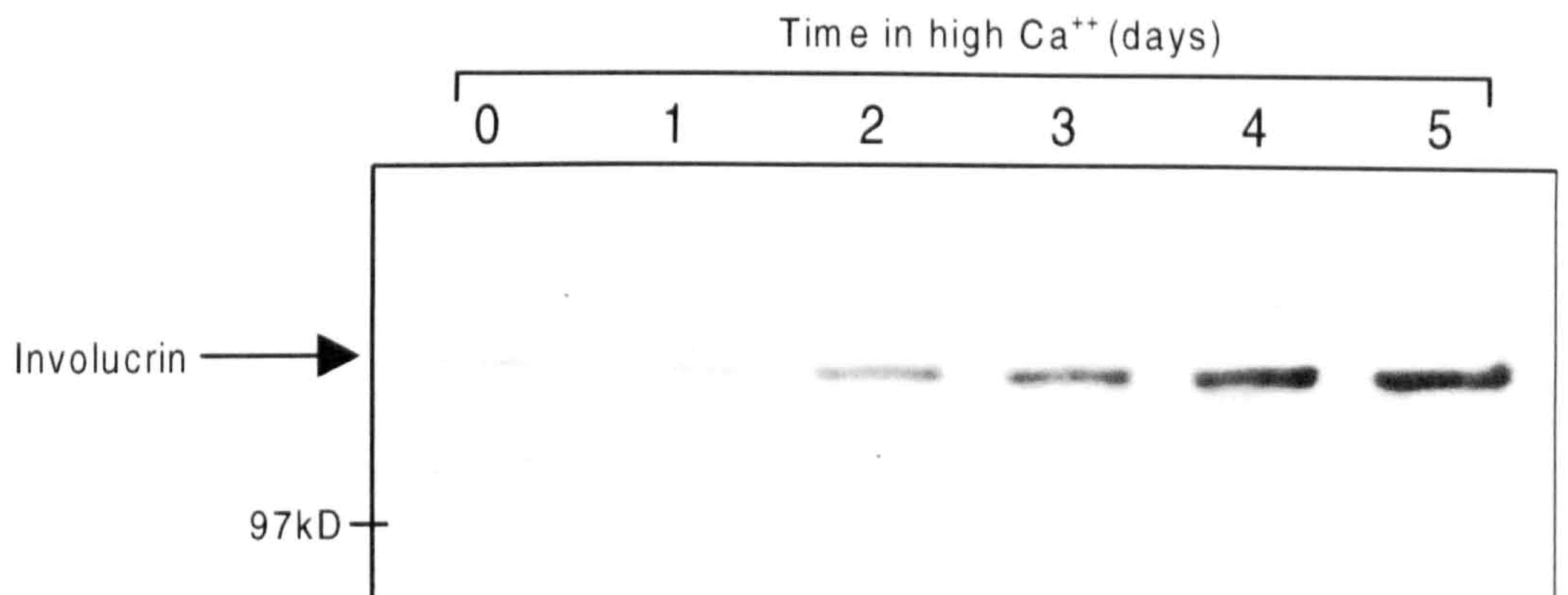


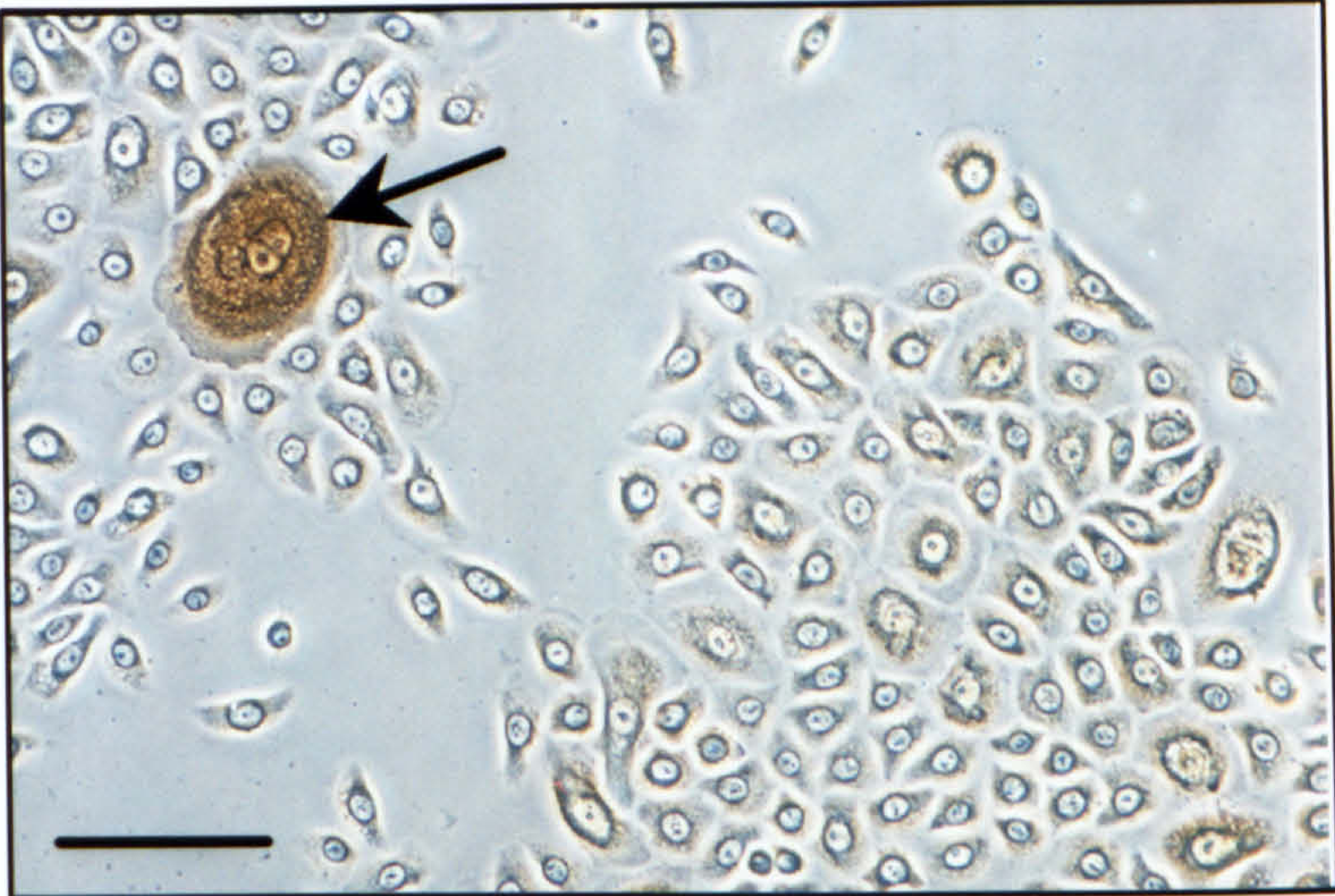
Figure 32

Figure 32
Prolonged high cell density induces involucrin expression

Phase-contrast photomicrographs of adherent keratinocytes grown in 0.03mM Ca^{++} at low density (*a*) or 5 days after reaching confluence (*b*) stained for involucrin by the immunoperoxidase method. The antiserum used was anti-involucrin rabbit polyclonal, diluted 1:5. Scale bars represent 100μm.

Prolonged high cell density induces involucrin expression

a. low extracellular calcium



b. high cell density, 5 days

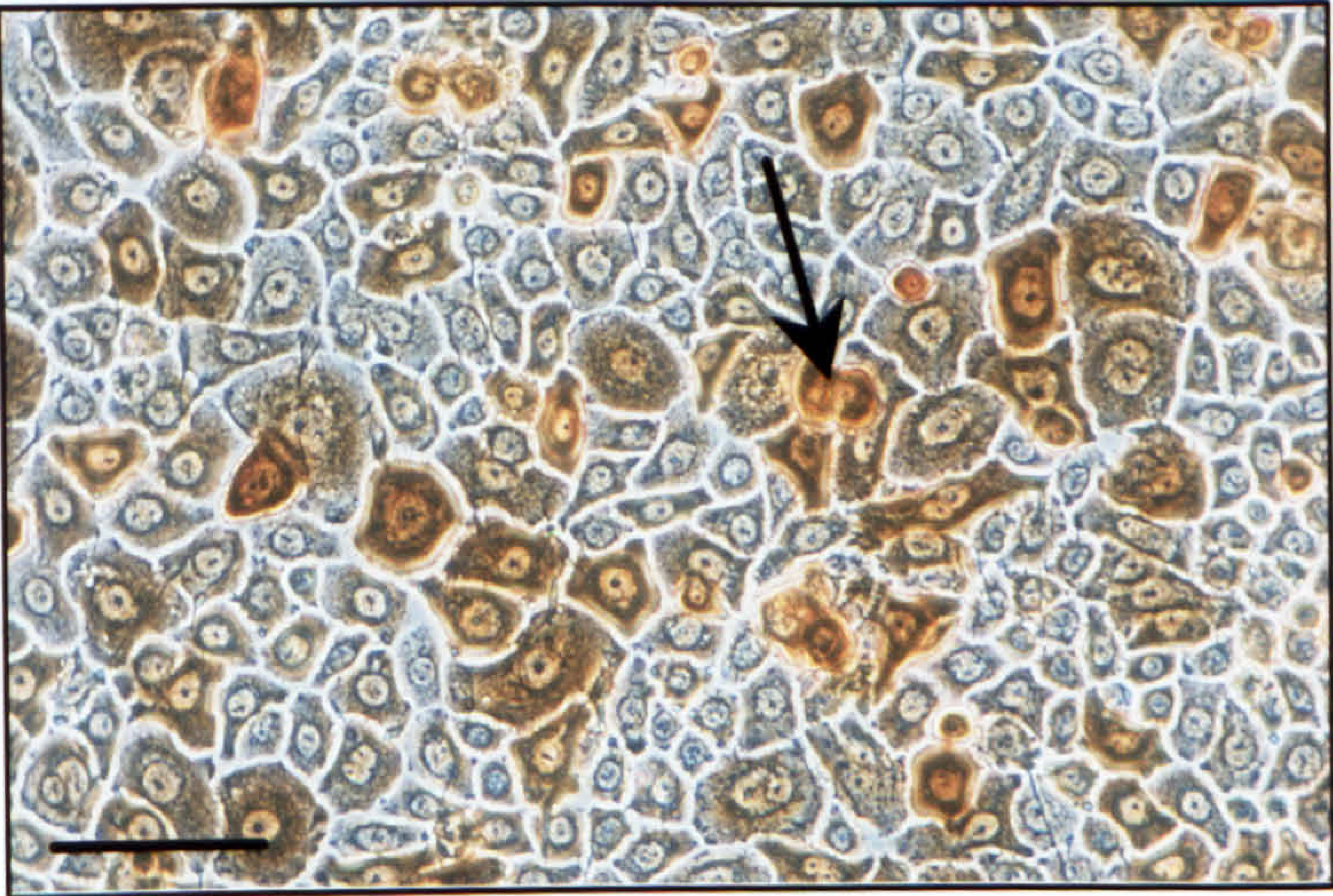
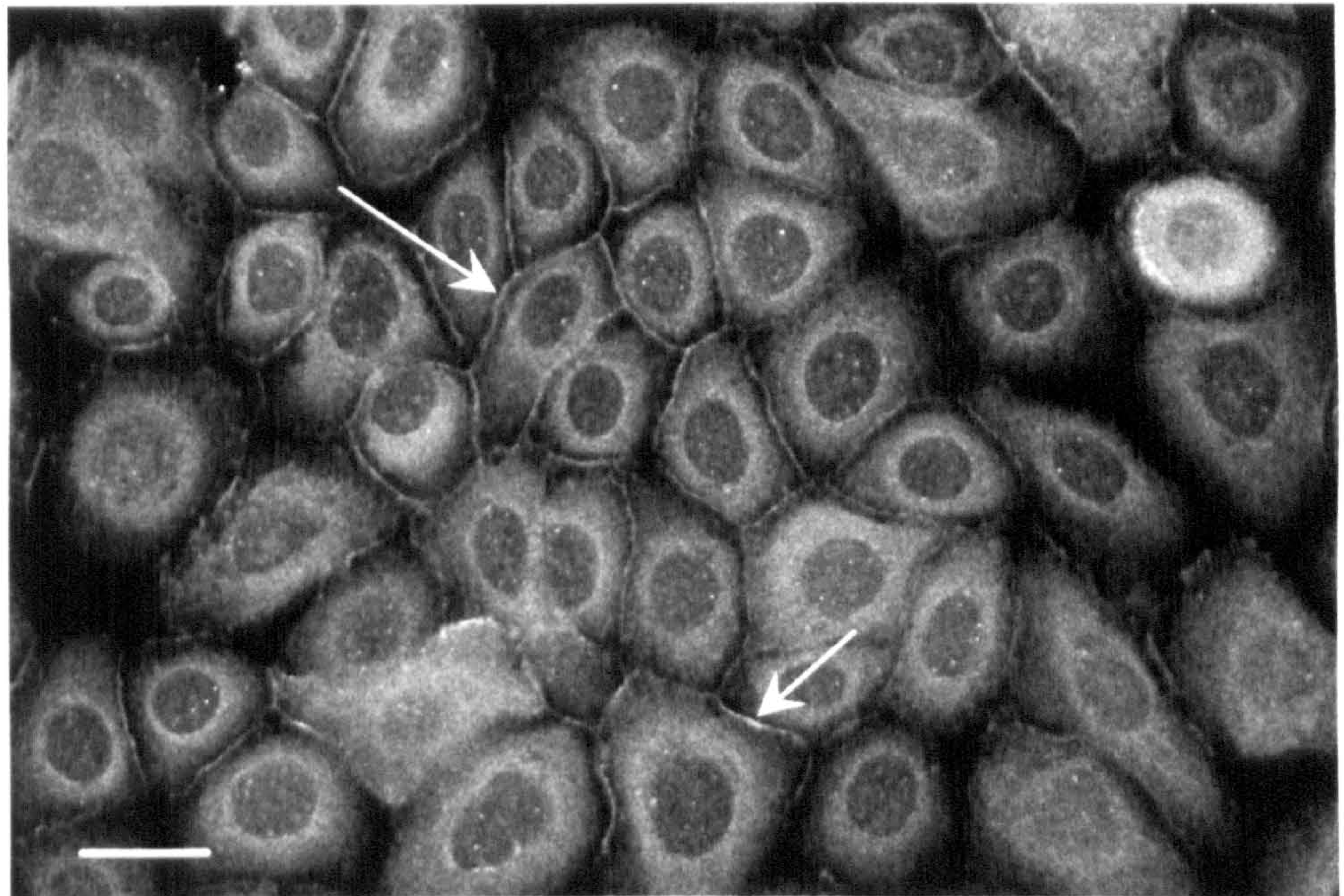


Figure 33

Figure 33
The localisation of E-cadherin in post-confluent cells maintained in low calcium

A confocal immunofluorescence image of post-confluent adherent keratinocytes stained for E-cadherin after growth in 0.03mM extracellular calcium. The antisera used was mouse anti-E-cadherin (clone #36) diluted 1:250. The scale bar represents 25μm.

The localisation of E-cadherin in post-confluent cells maintained in low calcium



8.5 Discussion

8.5.1 A potential role for the cadherins in differentiation induced by high cell density

Differentiation was induced by prolonged culture at high density in low extracellular Ca^{++}, ostensibly implying that the formation of Ca^{++}-dependent adhesions does not play a direct role in the initiation of differentiation by this mechanism. To our surprise, when cells grown to high density in 0.03mM Ca^{++} were stained for E-cadherin or the β_1, α_2 or α_3 integrin subunits, staining of the cell-cell contacts for all these proteins was observed (not shown for the integrins). This indicates that high extracellular Ca^{++} concentrations are not necessarily required for the formation of cadherin-mediated cell-cell adhesions, nor for the relocalisation of keratinocyte integrins to sites of cell-cell contact. In low extracellular Ca^{++}, the E-cadherin staining was less intense than was seen in cells switched to HKGM possibly indicating that the adhesions formed under high density (low Ca^{++}) conditions are weaker than those formed in HKGM, although this was not tested. Such adhesions may be stabilised by the restricted cell movement enforced by high cell density. Thus, two growth conditions which induce increased keratinocyte differentiation (high extracellular Ca^{++} and high cell density) both cause the redistribution of cadherins and integrins to cell-cell adhesions. This is consistent with a role for the formation of these adhesions and resultant intracellular signalling in mediating the induction of keratinocyte differentiation.

8.5.2 Cadherin-blocking peptides suppress calcium-induced differentiation

The experiments described in section 8.3 demonstrated that Ca^{++}-induced cell-cell adhesion was suppressed by a peptide analogous to the cadherin recognition domain. The synthetic peptide is likely to have blocked the functions of both E- and P-cadherin; studies using blocking antisera have shown that the function of both cadherins must be inhibited to prevent intercellular adhesion (Lewis *et al.,* 1994b). Furthermore, the induction of increased transglutaminase expression by high extracellular Ca^{++} was abrogated in the presence of the blocking peptide, suggesting that it is through the formation of cadherin-mediated adherens junctions that keratinocytes initiate differentiation *in vitro* in response to high extracellular Ca^{++} concentrations. However, treatment with the peptide will also indirectly suppress the formation of desmosomes

and perhaps other adhesive events that occur downstream of adherens junction assembly (O'Keefe *et al.,* 1987; Lewis *et al.,* 1994b). Therefore these experiments cannot completely exclude downstream events such as desmosome assembly or the changes in cell shape which occur in response to adherens junction assembly from playing some role in Ca^{++}-induced differentiation.

There are reports that murine keratinocytes sense extracellular calcium concentration through an extracellular divalent cation receptor (Filvaroff *et al.,* 1994), altering their intracellular Ca^{++} concentration in response to high extracellular Ca^{++} detected by the receptor. The data presented in section 8.3 are not consistent Ca^{++}-induced human keratinocyte differentiation being mediated in an adhesion-independent manner. Furthermore, the intracellular Ca^{++} concentration of human keratinocytes does not respond significantly to extracellular Ca^{++} (Watt *et al.,* 1991), indicating that Ca^{++} receptor-mediated responses are not likely to be important in the initiation of human *in vitro* keratinocyte differentiation.

A possible interpretation of the effects of the cadherin-blocking peptides on Ca^{++}-induced differentiation is that the peptide causes the selective detachment of differentiated cells, leaving only the undifferentiated cells attached to the substrate. This could, in theory, occur as cells maintained in high extracellular Ca^{++} are apparently unable to form adhesions with the extracellular matrix once they have begun to differentiate and may require cadherin function for their retention in the suprabasal layers of the culture (Adams and Watt, 1990). However, this lost adhesiveness to the ECM only occurs in differentiating cells with functional cadherins and is therefore likely to be a downstream event of the formation of adherens junctions (Hodivala and Watt, 1994). Thus, it is unclear as to whether integrin loss will occur in cells switched to high extracellular Ca^{++} in the presence of cadherin blocking peptide. Attempts to resolve this issue by measuring cell detachment in the presence and absence of cadherin blocking peptide indicated that there was no appreciable cell detachment (not shown). Thus, interfering with cadherin-mediated cell-cell adhesion suppressed *in vitro* Ca^{++}-induced keratinocyte differentiation by a mechanism which did not involve substantial detachment of differentiated cells.

8.5.3 Clustering cell-surface E-cadherin induces increased differentiation-specific protein expression

The cadherin and integrin clustering, which should mimic some features of intercellular adhesion in low extracellular Ca^{++}, complement the experiments using blocking peptides. None of the antisera used induced cell detachment, including mAb 13 (anti-β_1-integrin), indicating that keratinocyte-matrix adhesions involve other integrins additional to those containing the β_1-subunit. These β_1-independent adhesions are likely to include the stable anchoring contact (SAC) described by Carter *et al.*, (1990b) which contains hemidesmosomal components.

When the cells were treated to cluster E-cadherin, a substantially increased proportion were expressing involucrin and TGk four days later. This shows that the process of E-cadherin clustering is sufficient to induce the expression of differentiation-specific proteins in a significant proportion of the cells, a phenomenon that was not observed when β_1-integrin was clustered. However, it is not clear how closely antibody-mediated clustering mimics normal intercellular adhesions. When a cadherin-mediated adherens junction forms, the initial adhesion is strengthened by the lateral clustering of additional cadherin molecules in the membrane, increasing the number of molecules participating in the adhesion (Tomschy *et al.*, 1996). It is possible that it is this process which is mimicked by the antibody-mediated clustering procedure. However, antibody-mediated clustering may not fully recreate all aspects of the formation of an adherens junction, e.g. conformational changes in the cadherin resulting from Ca^{++} binding and binding to other cadherin molecules on adjacent cells. The antibody used for these clustering experiments (DECMA-1) is known to interact with a membrane-proximal region of the extracellular domain of E-cadherin but the effects this binding has on conformation have not been determined (Ozawa *et al.*, 1990). It is not clear what type of integrin-mediated adhesion is mimicked by clustering since keratinocytes form two different types of adhesion involving β_1-integrin (cell-cell and cell-matrix). Both types of adhesion are likely to result in the clustering of the β_1-integrin subunit into localised areas of high density although the conformation of the integrin may be different in the two types of adhesion. In cell-ECM adhesions, the integrin receptors bind to extracellular matrix components (collagen and laminin 5 for the principal $\alpha_2\beta_1$ and $\alpha_3\beta_1$ keratinocyte integrins) but in cell-cell adhesions these integrins are capable of directly

associating with one another (Symington *et al.*, 1993). However, in fibroblasts treated to cluster the β_1-integrin subunit, integrin clustering is thought to mimic adhesion to the extracellular matrix (Kornberg *et al.*, 1991).

There are two classical cadherins present in human keratinocytes (section 2.2.2) but only one was clustered to investigate its role in the induction of differentiation, leaving the role of P-cadherin in this process unexplored. Unfortunately, we could not obtain sufficient quantities of a suitable anti-P-cadherin antiserum to attempt these experiments. However, the E-cadherin clustering experiments showed that clustering this protein on the cell surface was sufficient to induce the expression of two differentiation-specific proteins at low extracellular Ca^{++} concentrations.

8.6 SUMMARY

This group of experiments produced data supporting a role for E-cadherin in mediating at least some aspects of *in vitro* differentiation. This is in agreement with the extensive body of literature documenting a strong correlation between the loss of E-cadherin and impaired differentiation in a number of different types of carcinoma (section 2.2.2; reviewed in Birchmeier and Behrens, 1994).

The antibody-mediated clustering experiments demonstrated that clustering cell surface β_1-integrin had no significant effect on the proportion of cells expressing involucrin. However, it is clear that the procedure of clustering a cell adhesion molecule in this way does not artifactually increase the proportion of cells expressing differentiation-specific proteins, indicating that the effects observed when E-cadherin was clustered are specific to E-cadherin.

CHAPTER 9

THE Src-FAMILY KINASES AND KERATINOCYTE DIFFERENTIATION

9.1 PURPOSE

The initial aim of this study was to determine if there was a role for the Src-family kinases in Ca^{++}-induced *in vitro* human keratinocyte differentiation. A small number of publications have shown that this family of tyrosine kinases may play a role in the control of differentiation although their precise function in normal keratinocytes is unclear. There are also reports that members of the Src-family are involved in controlling the stability of the cadherin-mediated adhesions in some epithelial cell types (Takeda *et al.,* 1995). In chapters 6 and 8, data consistent with a role for the cadherins in the initiation of differentiation were presented, thus indicating that the modulation of these adhesions, for example by c-Src, could have profound effects on keratinocyte behaviour. The experiments described in this chapter investigate the Src kinases during *in vitro* Ca^{++}-induced keratinocyte differentiation.

9.2 THE EFFECTS OF EXTRACELLULAR CALCIUM CONCENTRATION ON MEMBERS OF THE SRC-FAMILY

To begin this group of experiments on the Src kinases, the subcellular localisation, protein levels and kinase activities of c-Src, Fyn and c-Yes were determined in cells maintained in 0.03mM extracellular Ca^{++} or shifted to 1mM extracellular Ca^{++}.

9.2.1 High extracellular calcium alters the subcellular localisation of c-Src, Fyn and c-Yes

To determine the subcellular localisations of c-Src, Fyn and c-Yes, subconfluent keratinocytes were maintained on glass chamber slides in LKGM and switched to HKGM for 4 or 24 hours. The cells were then immunostained for c-Src, Fyn or c-Yes.

Figure 34 shows keratinocytes stained for c-Src. When the cells were maintained in 0.03mM extracellular Ca^{++} (Figure 34 *a*), the staining pattern for c-Src was diffuse. Under identical growth conditions the same was also true for c-Yes (Figure 35 *a*) although occasional punctate staining at the level of cell-substrate contact was also seen, perhaps corresponding to cell-ECM adhesions (not shown). However, when the cells were switched to 1mM extracellular Ca^{++}, changes in the subcellular distribution of both kinases were apparent. After four hours in HKGM, c-Src was relocalised to areas of cell-cell contact (Figure 34 *b*, arrow) in a manner similar to that seen for E-cadherin under the same conditions (Figure 16 *b*). At this time, the staining pattern resembled a series of lines inserting into the areas of cell-cell contact, suggesting that c-Src may be arranged along fibrous structures during its translocation into the adhesion, as described for E-cadherin in section 6.5.1. For c-Yes, high extracellular Ca^{++} also induced the relocalisation of this protein into the areas of cell-cell contact (Figure 35 *b*, arrow), an event that was sensitive to cytochalasin D but not nocodazole (Figure 35 *d* and not shown).

After 24 hours in high extracellular Ca^{++}, both c-Src and c-Yes were clearly localised to areas of cell-cell contact (Figures 34 *c* and 35 *c*, arrows), as has previously been described for rat keratinocytes (Tsukita *et al.*, 1991). Furthermore, similar to the cadherins, the staining pattern after the longer incubation in high extracellular Ca^{++} appeared as a narrower band in the cell-cell contact zone than at the earlier times after switching to HKGM. Unfortunately the anti-Fyn polyclonal antiserum gave only weak staining when used for immunofluorescence microscopy, although occasional staining of cell-cell contact areas was seen in cells that had been maintained in high extracellular Ca^{++} (not shown). Thus, high extracellular Ca^{++} induced the relocalisation of c-Src, c-Yes and probably also Fyn to sites of cell-cell contact. The sensitivity of c-Yes relocalisation to the actin-disrupting drug cytochalasin D indicates that this process may share a common actin-dependent mechanism with E-cadherin translocation.

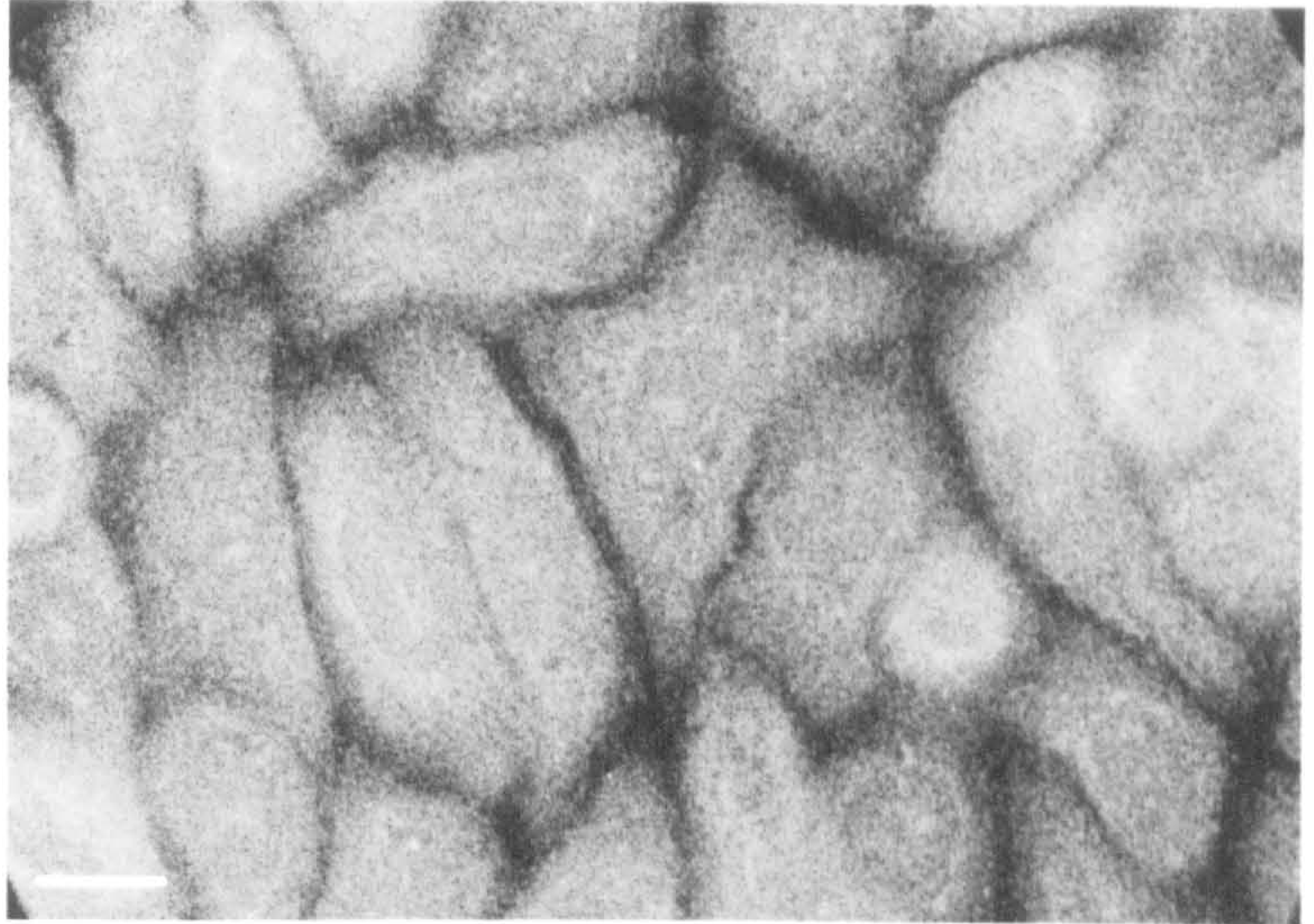
Figure 34

Figure 34
High extracellular calcium induces c-Src relocalisation

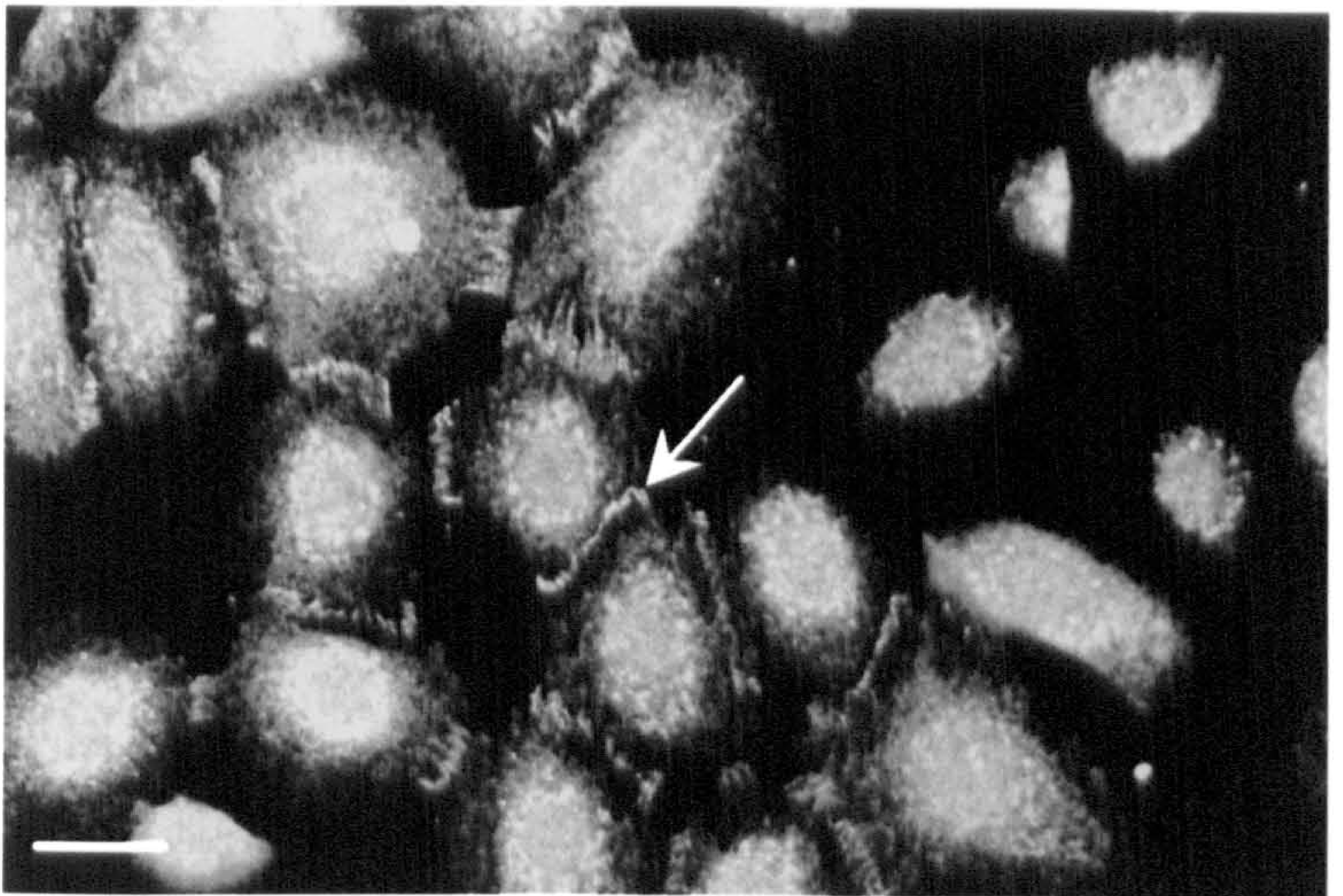
Confocal immunofluorescence micrographs of adherent keratinocytes stained for c-Src after maintenance in 0.03mM extracellular Ca^{++} (*a*) or after 4 (*b*) and 24 hours (*c*) in 1mM extracellular Ca^{++}. The antiserum used was anti-c-Src, mAb 327 ascites (a generous gift from Dr. S. Simon, Salk Institute) diluted 1:100 visualised with FITC-conjugated anti-mouse IgG diluted 1:100. The scale bars represent 25μm.

High extracellular calcium induces c-Src relocalisation

a. low extracellular calcium



b. high extracellular calcium, 4 hours



c. high extracellular calcium, 24 hours

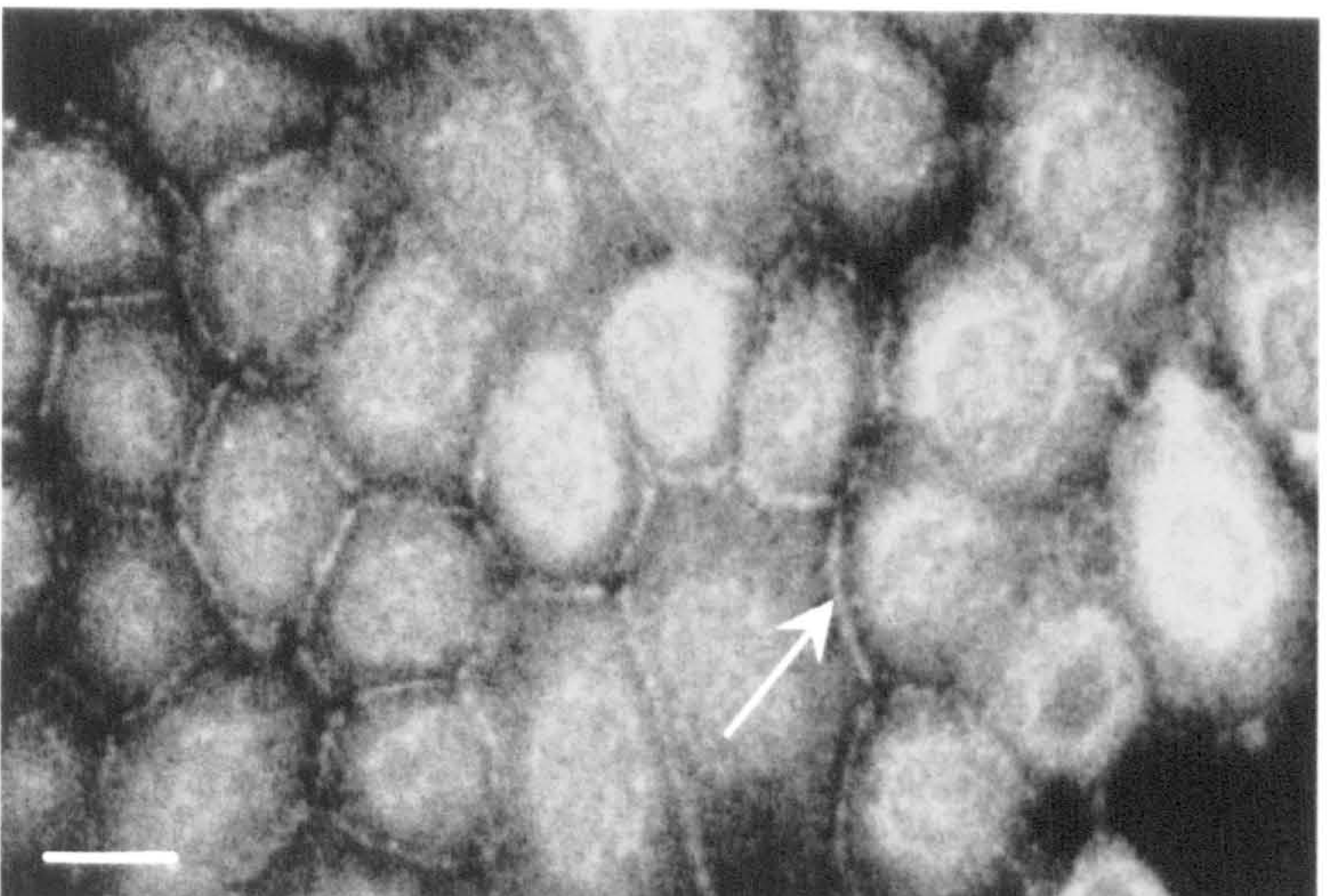


Figure 35

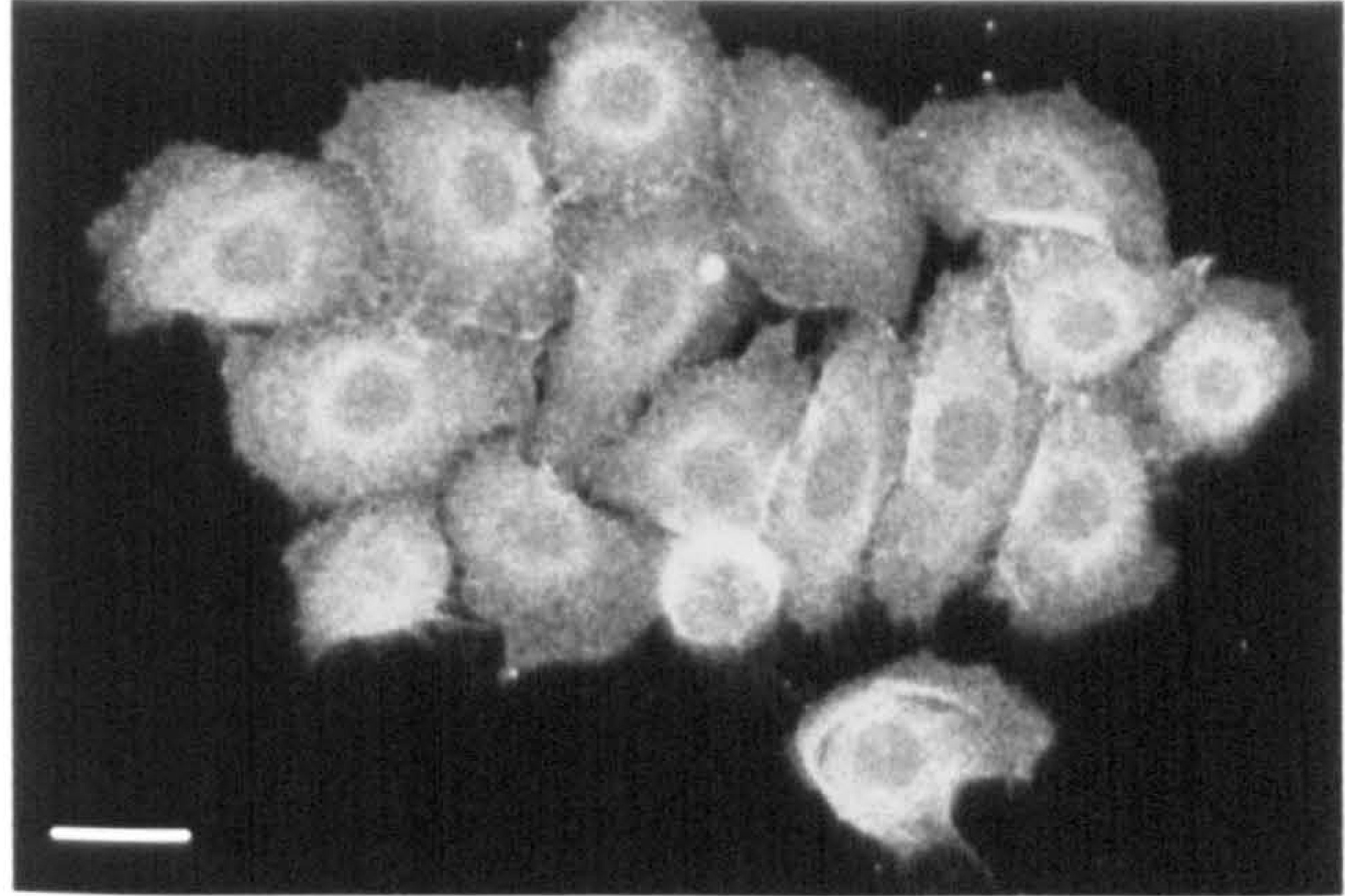
Figure 35

High extracellular calcium induces c-Yes relocalisation

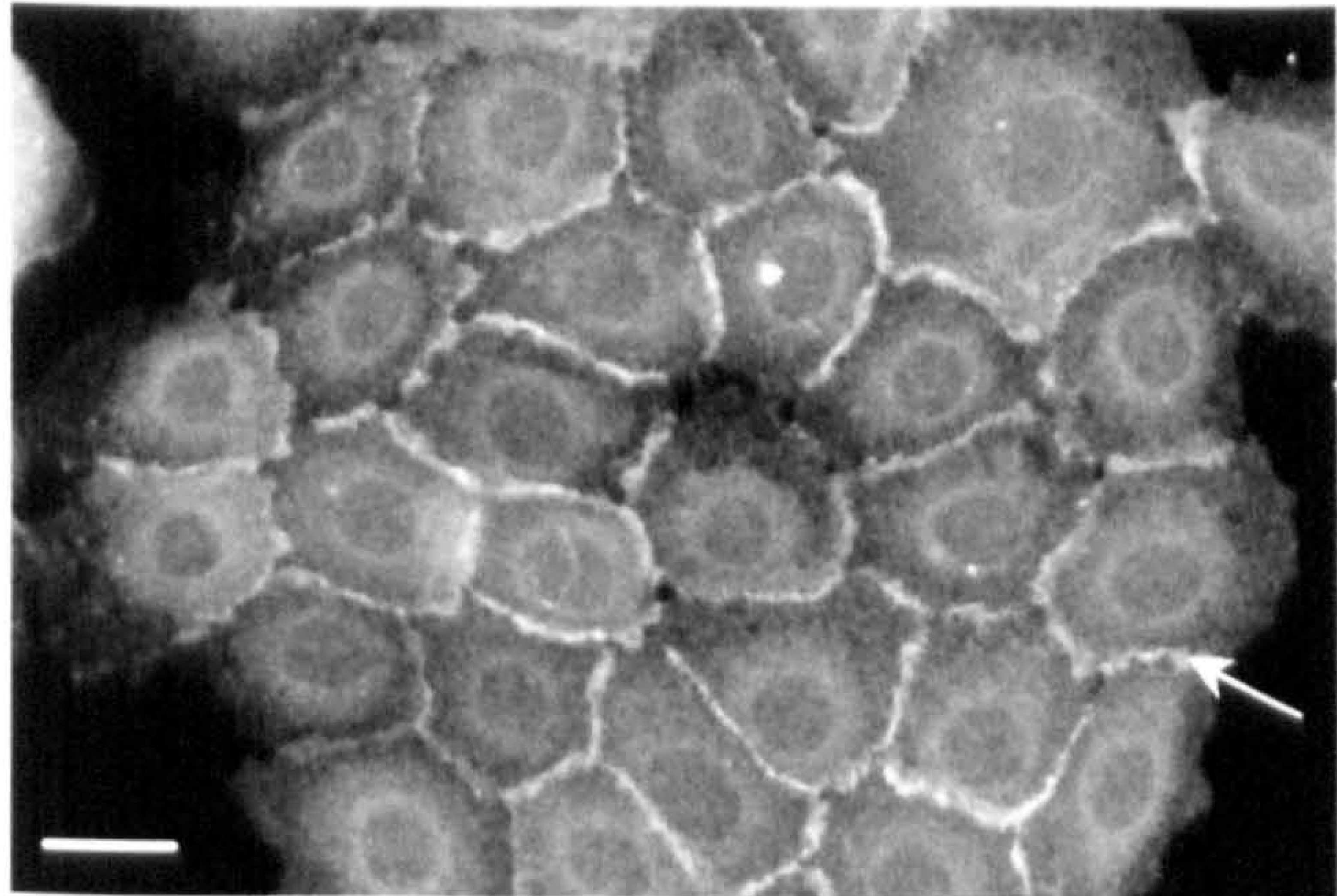
Confocal immunofluorescence micrographs of adherent keratinocytes stained for c-Yes after maintenance in 0.03mM extracellular Ca^{++} (*a*), after maintenance in 1mM extracellular Ca^{++} for 4 (*b*), 24 hours (*c*) and after 4 hours in 1mM extracellular Ca^{++} in the presence of 5µg/ml cytochalasin D (*d*) or 2.5µg/ml nocodazole (*e*). The antiserum used was anti-c-Yes, mAb clone #1, diluted 1:100 visualised with FITC-conjugated anti-mouse IgG diluted 1:100. The scale bars represent 25µm.

High extracellular calcium induces c-Yes relocalisation

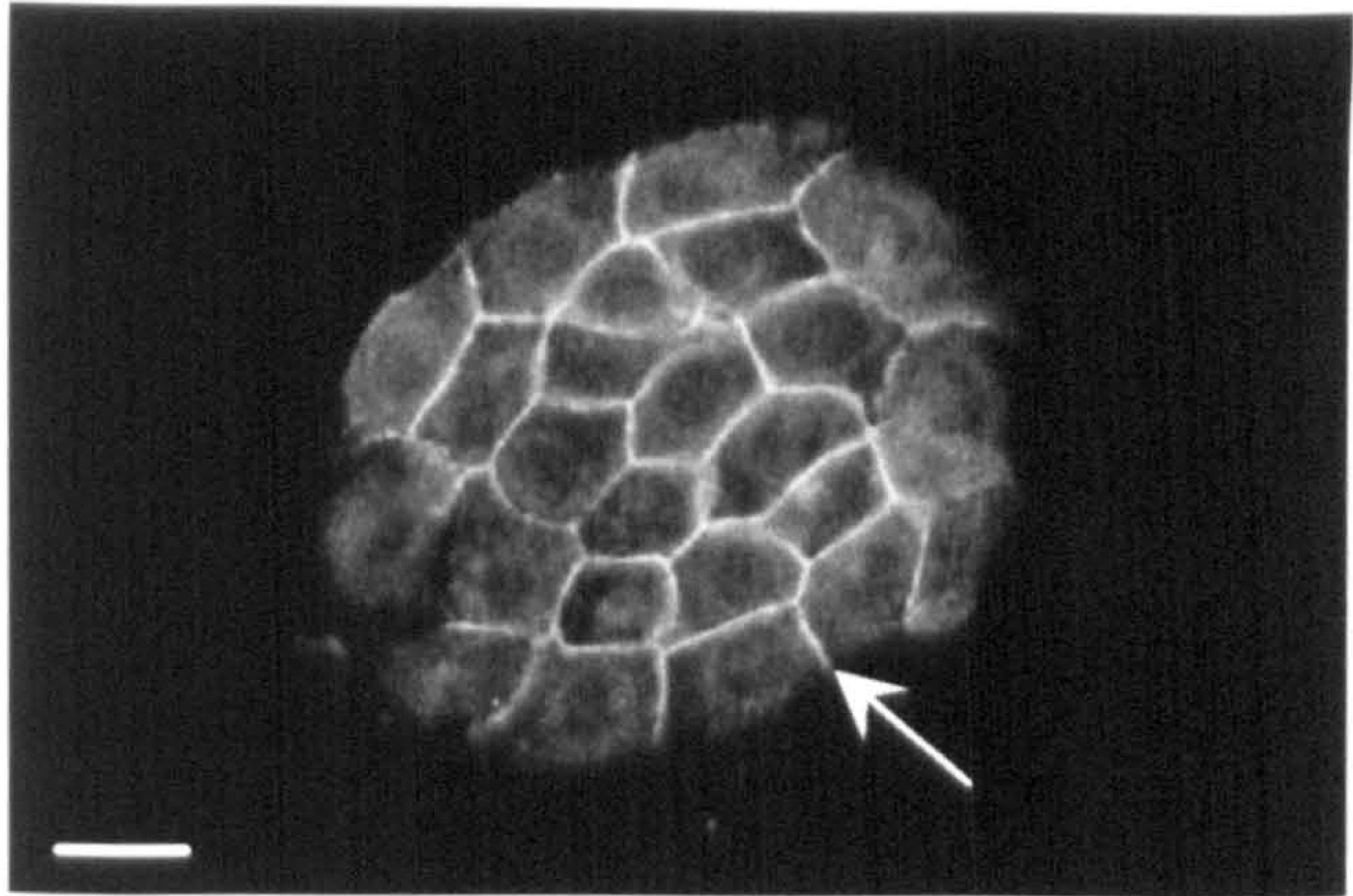
a. low extracellular calcium



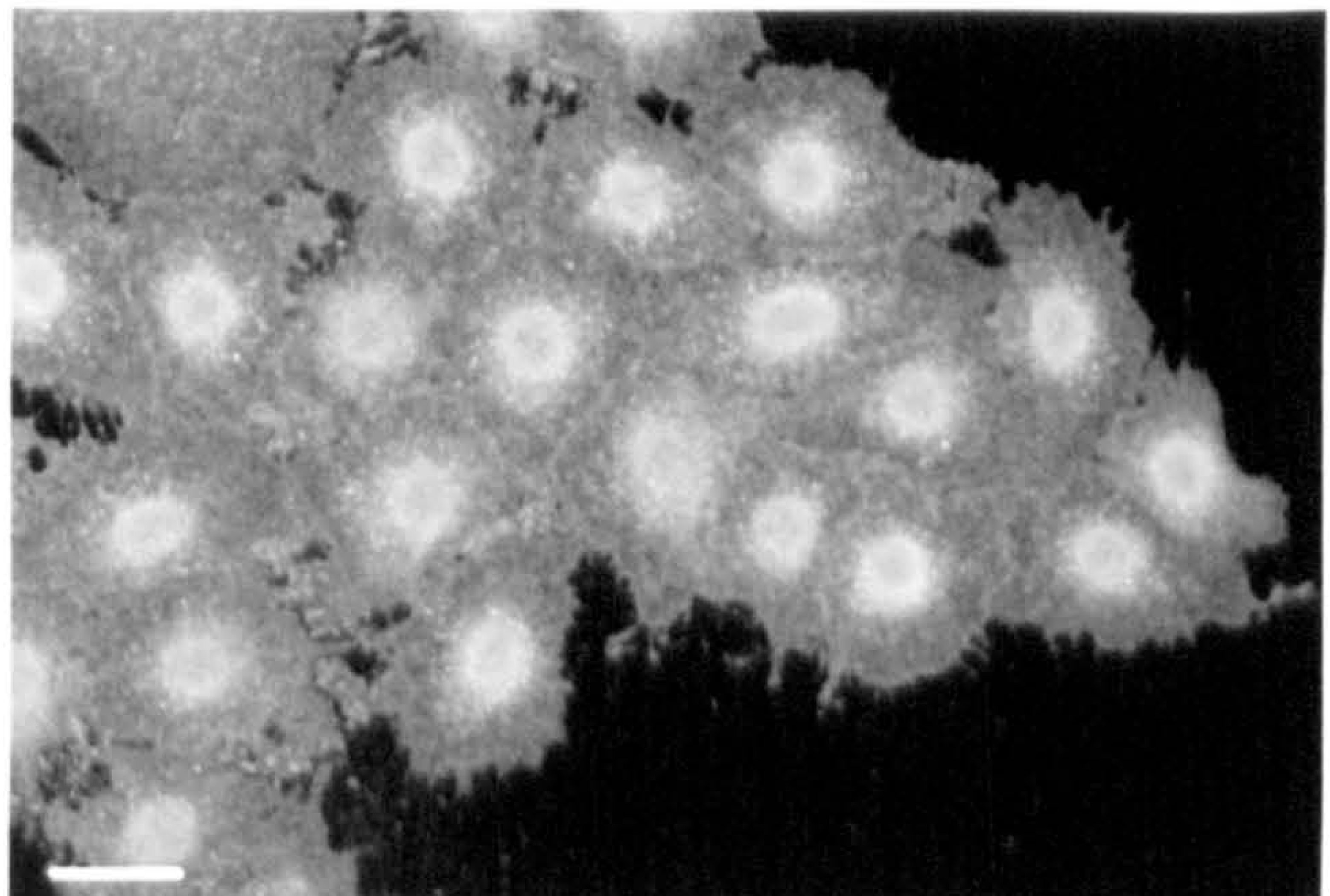
b. high extracellular calcium, 4 hours



c. high extracellular calcium, 24 hours



d. high extracellular calcium, 4 hours + cytochalasin D



9.2.2 Extracellular calcium does not have an appreciable effect on c-Src, Fyn and c-Yes abundance

Having determined the subcellular localisation of members of the Src-family kinases, the abundance of these kinases in cells grown under different extracellular Ca^{++} conditions were then determined by immunoblotting. Subconfluent adherent keratinocytes were maintained in LKGM or switched to HKGM for a duration of up to 5 days before lysing in RIPA buffer and immunoblotting (Figure 36).

When keratinocytes were maintained in 0.03mM extracellular Ca^{++}, they expressed readily detectable quantities of c-Src (Figure 36 *a*), Fyn (*b*) and c-Yes (*c*), shown in the t = 0 lanes of the blots. Upon switching to HKGM, no obvious consistent changes in the protein levels or electrophoretic mobility of any of these three kinases were seen (t = 1 day) despite the changes in subcellular localisation that took place during this time (section 9.2.1). Similarly, extended incubation in high extracellular Ca^{++} for up to 5 days also failed to induce any major changes in the amounts of these proteins that were detected by immunoblotting (t = 2-5 days) although a transient and modest increase in c-Src abundance and a similarly small increase in c-Yes abundance were seen in this experiment. No changes in the electrophoretic mobility of any of these kinases was induced by increasing the extracellular Ca^{++} concentration.

In the epidermis, the levels of c-Yes decrease as the keratinocytes enter the spinous layer whereas the levels of c-Src are not appreciably altered (Krueger *et al.,* 1991; Zhao *et al.,* 1992). The importance of this decrease in c-Yes abundance during differentiation is not clear as it was not seen in the keratinocyte populations induced to differentiate with high extracellular Ca^{++}. Thus, high extracellular Ca^{++} and subsequent *in vitro* keratinocyte differentiation did not have an appreciable effect on the abundance of c-Src, Fyn or c-Yes.

Figure 36

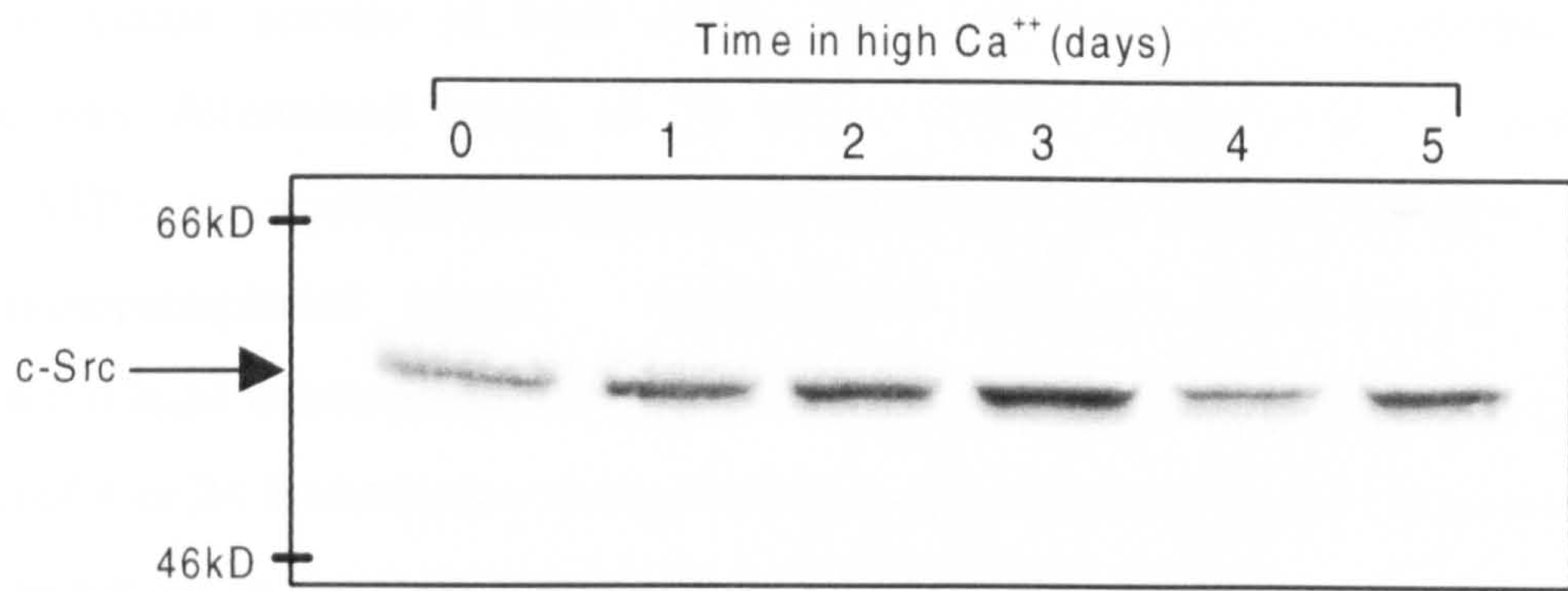
Figure 36

The effects of extracellular calcium on Src-family kinase abundance

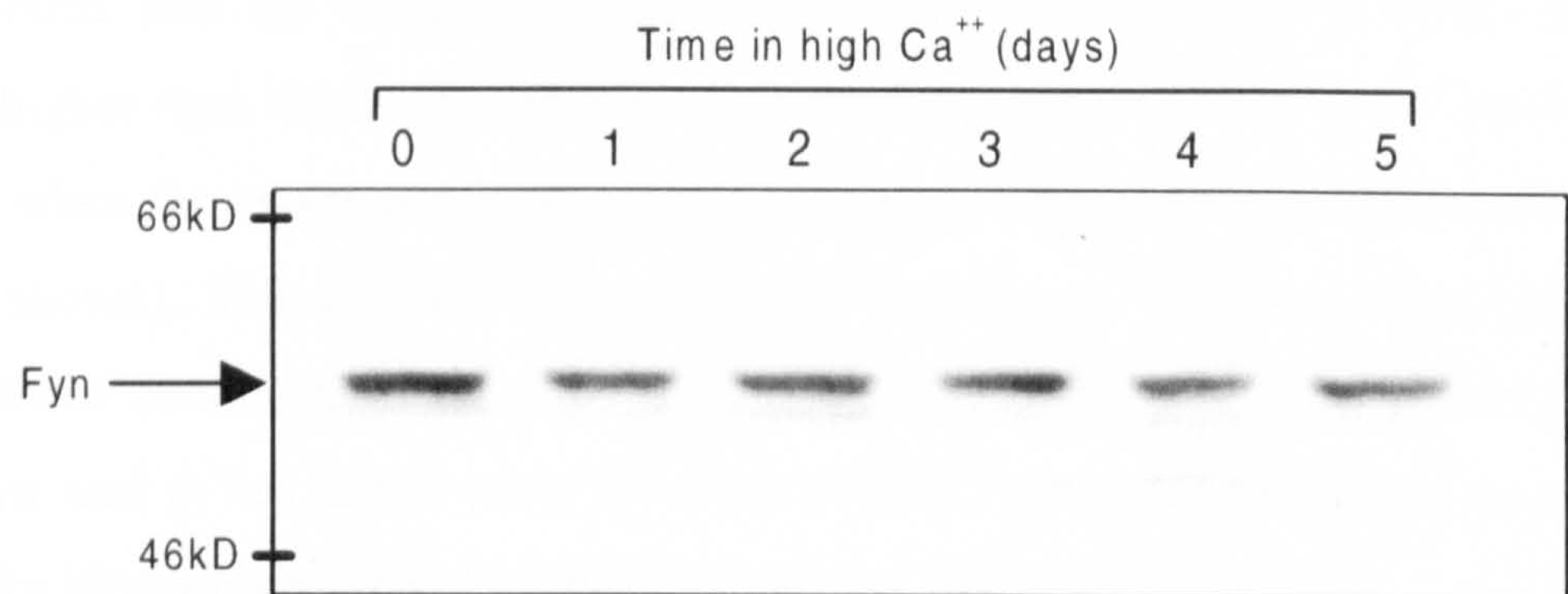
Immunoblots for c-Src (*a*), Fyn (*b*) and c-Yes (*c*) prepared using proteins extracted using RIPA lysis buffer from keratinocytes maintained in 0.03mM or switched to 1mM extracellular Ca^{++} for the indicated times. The antisera used were mAb 327 diluted 1:1000, anti-Fyn rabbit polyclonal diluted 1:500 and anti-c-Yes clone #1 diluted 1:1000.

The effects of extracellular calcium on Src-family kinase abundance

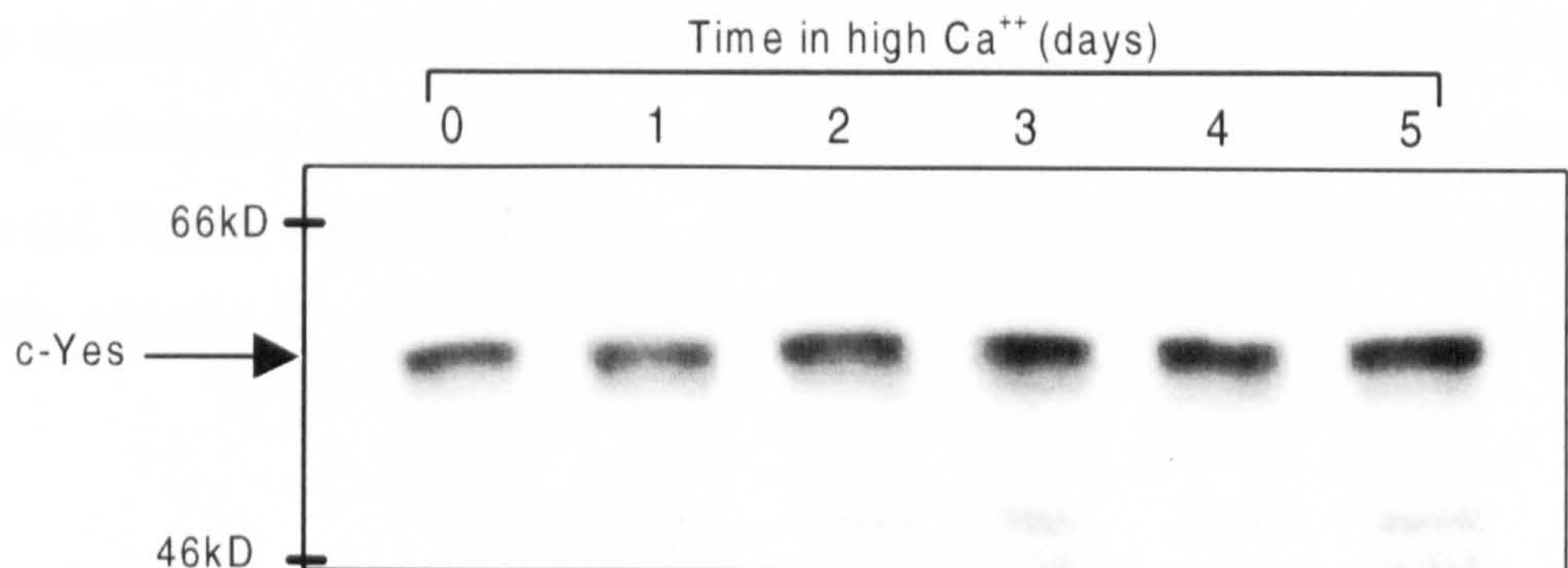
***a.* c-Src immunoblot**



***b.* Fyn immunoblot**



***c.* c-Yes immunoblot**



9.2.3 Extracellular calcium does not have an appreciable effect on Src-family kinase activity

The data presented in section 9.2.2 showed that increasing the extracellular Ca^{++} concentration did not affect the amounts of c-Src, Fyn and c-Yes present in the cells. However many of the biological functions of the Src kinases require their tyrosine kinase activity which may vary without alterations in the abundance of the protein. Therefore, the kinase activity of each of the three Src-family kinases present in keratinocytes was determined using an *in vitro* tyrosine kinase assay in which radiolabelled ATP is incorporated into an artificial substrate polypeptide (poly(Glu,Tyr)) by the immunoprecipitated kinase. Subconfluent adherent keratinocytes were maintained in 0.03mM extracellular Ca^{++} or were switched to 1mM extracellular Ca^{++} for a duration of 4 or 24 hours before lysing in CSK buffer and immunoprecipitating the required kinase for use in the assay as described in sections 5.8 and 5.9.

The kinase activities of c-Src, Fyn and c-Yes extracted from keratinocytes grown in different extracellular Ca^{++} conditions are summarised in Figure 37. Kinase activity is expressed in counts per minute (cpm) corresponding to the amount of radiolabelled ATP incorporated into the exogenous substrate. The kinase activity of c-Src was considerably higher than those of Fyn and c-Yes as determined by this assay and this was also true when the experiment was repeated using a commercial Src-family kinase assay kit (not shown). This indicates that c-Src has a higher specific activity than Fyn or c-Yes under these conditions. The reason for the consistently high activity of c-Src relative to Fyn and c-Yes is not clear but may reflect a genuine difference or may indicate that the kinases have different affinities for the substrate polypeptide.

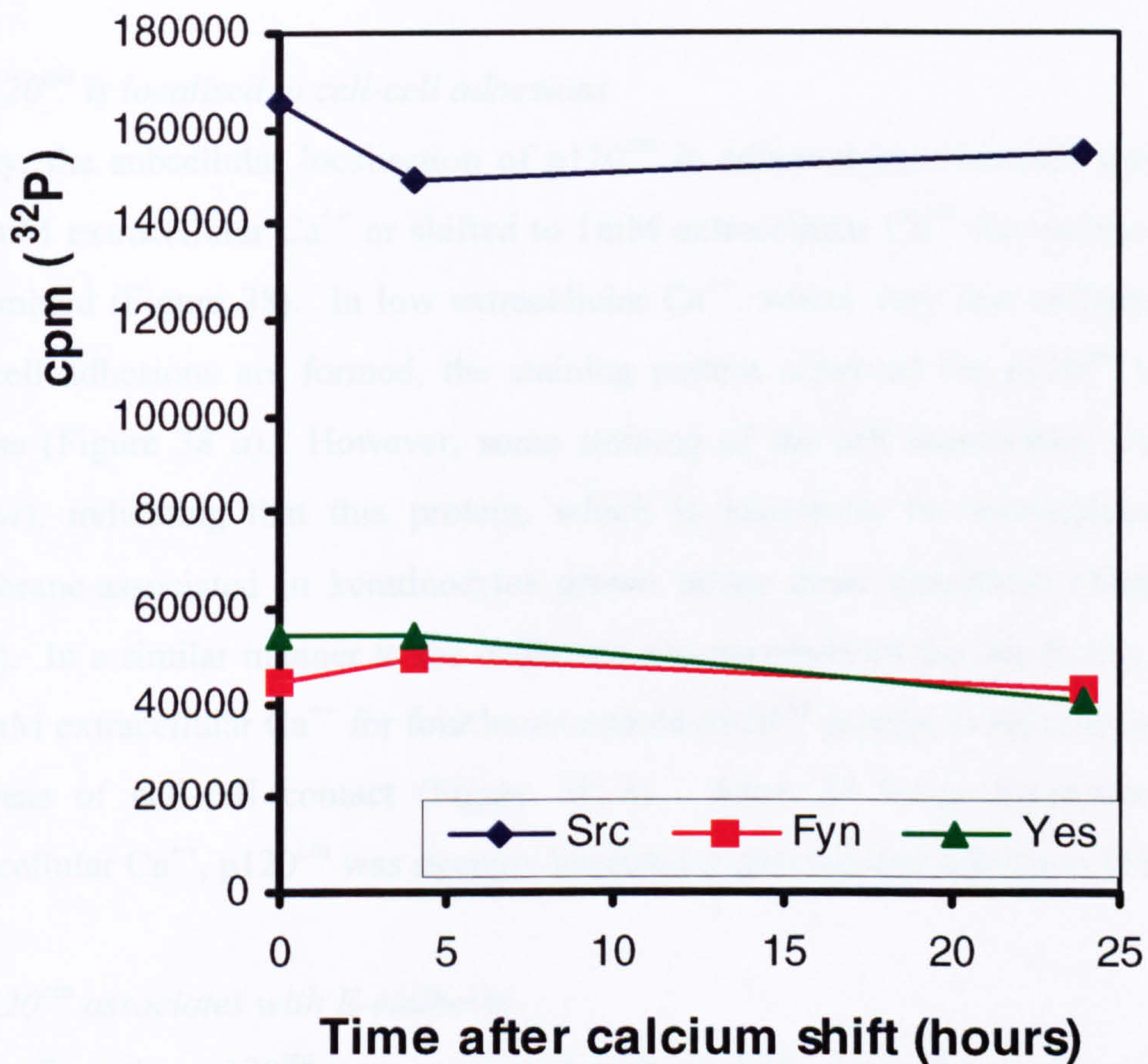
The data presented in Figure 37 show that switching to 1mM extracellular Ca^{++} had very little effect on the kinase activity of the three Src kinases. A slight decrease in c-Src activity was seen but the changes in the activity of Fyn and c-Yes were modest and probably not significant. However, when using assays of this type to measure tyrosine kinase activity, alterations in kinase activity with profound biological effects can appear insignificant (M. Frame, Beatson Institute). Thus, the possibility that localised changes in the specific activity of c-Src, Fyn or c-Yes are taking place cannot be ruled out.

Figure 37

Figure 37
The effects of extracellular calcium concentration on Src-family kinase activity

Summary of the data obtained from a series of *in vitro* tyrosine kinase assays measuring the activity of c-Src (shown in blue), Fyn (red) and c-Yes (green) extracted from cells maintained in 0.03mM extracellular Ca^{++} (t = 0) and after 4 and 24 hours in 1mM extracellular Ca^{++}.

The effects of extracellular calcium on Src-family kinase activity



9.2.4 Extracellular calcium alters the tyrosine phosphorylation of $p120^{ctn}$

The data presented in section 9.2.1 suggest that members of the Src-family kinases may play a role at the sites of cell-cell adhesion. The absence of any detectable changes in the activity of these kinases (section 9.2.2) suggests that any changes in kinase activity are too small to be detected by the assay. In order to determine the activity of the Src-family kinases localised to cell-cell adhesions, the subcellular localisation and tyrosine phosphorylation status of the cadherin-associated Src-substrate $p120^{ctn}$, previously known as $p120^{cas}$ (section 2.2.2), were investigated.

A. $p120^{ctn}$ is localised to cell-cell adhesions

Firstly, the subcellular localisation of $p120^{ctn}$ in adherent keratinocytes maintained in 0.03mM extracellular Ca^{++} or shifted to 1mM extracellular Ca^{++} for various times was determined (Figure 38). In low extracellular Ca^{++}, where very few cadherin-mediated cell-cell adhesions are formed, the staining pattern observed for $p120^{ctn}$ was largely diffuse (Figure 38 *a*). However, some staining of the cell membranes was apparent (arrow), indicating that this protein, which is known to be myristylated, may be membrane-associated in keratinocytes grown under these conditions (Kanner *et al.*, 1991). In a similar manner to the cadherins and members of the Src-family, switching to 1mM extracellular Ca^{++} for four hours caused $p120^{ctn}$ protein to become concentrated to areas of cell-cell contact (Figure 38 *b*). After 24 hours incubation in 1mM extracellular Ca^{++}, $p120^{ctn}$ was strongly localised to the cell-cell adhesions (Figure 38 *c*).

B. $p120^{ctn}$ associates with E-cadherin

To confirm that $p120^{ctn}$ was associated with E-cadherin in these cells, $p120^{ctn}$ was immunoprecipitated from keratinocytes maintained in LKGM or switched to 1mM extracellular Ca^{++} for 4 or 24 hours. These immunoprecipitates were washed extensively with RIPA buffer and immunoblotted for E-cadherin (Figure 39 *a*) and subsequently for $p120^{ctn}$ (Figure 39 *b*). Immunoprecipitations performed using non-immune normal mouse IgG did not contain detectable amounts of E-cadherin or $p120^{ctn}$ (Figure 39, control). However, the $p120^{ctn}$ immunoprecipitates contained E-cadherin and $p120^{ctn}$. Thus, $p120^{ctn}$ was physically associated with E-cadherin in keratinocytes and was also present at the intercellular adhesions at the same time as E-cadherin,

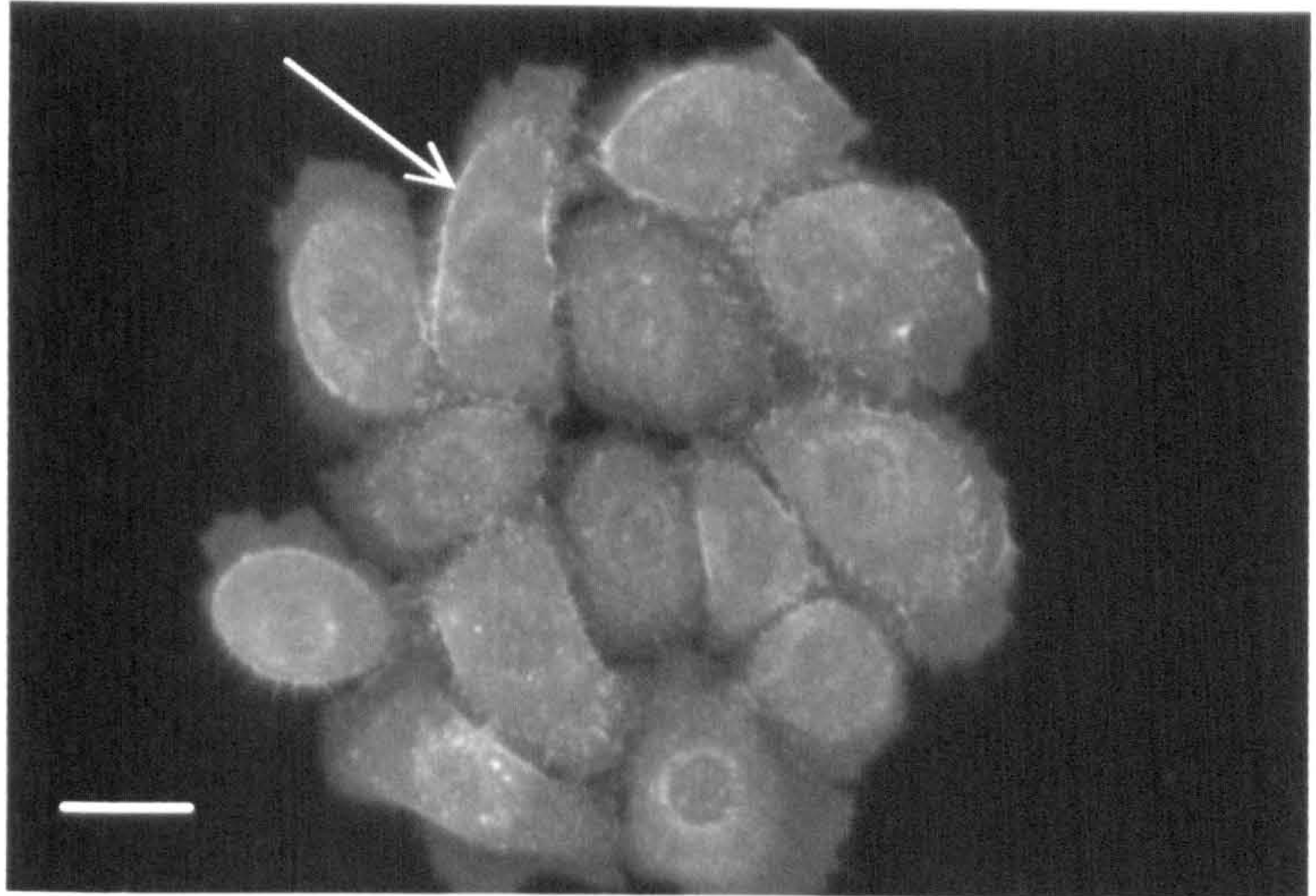
Figure 38

Figure 38
High extracellular Ca^{++} induces $p120^{ctn}$ relocalisation

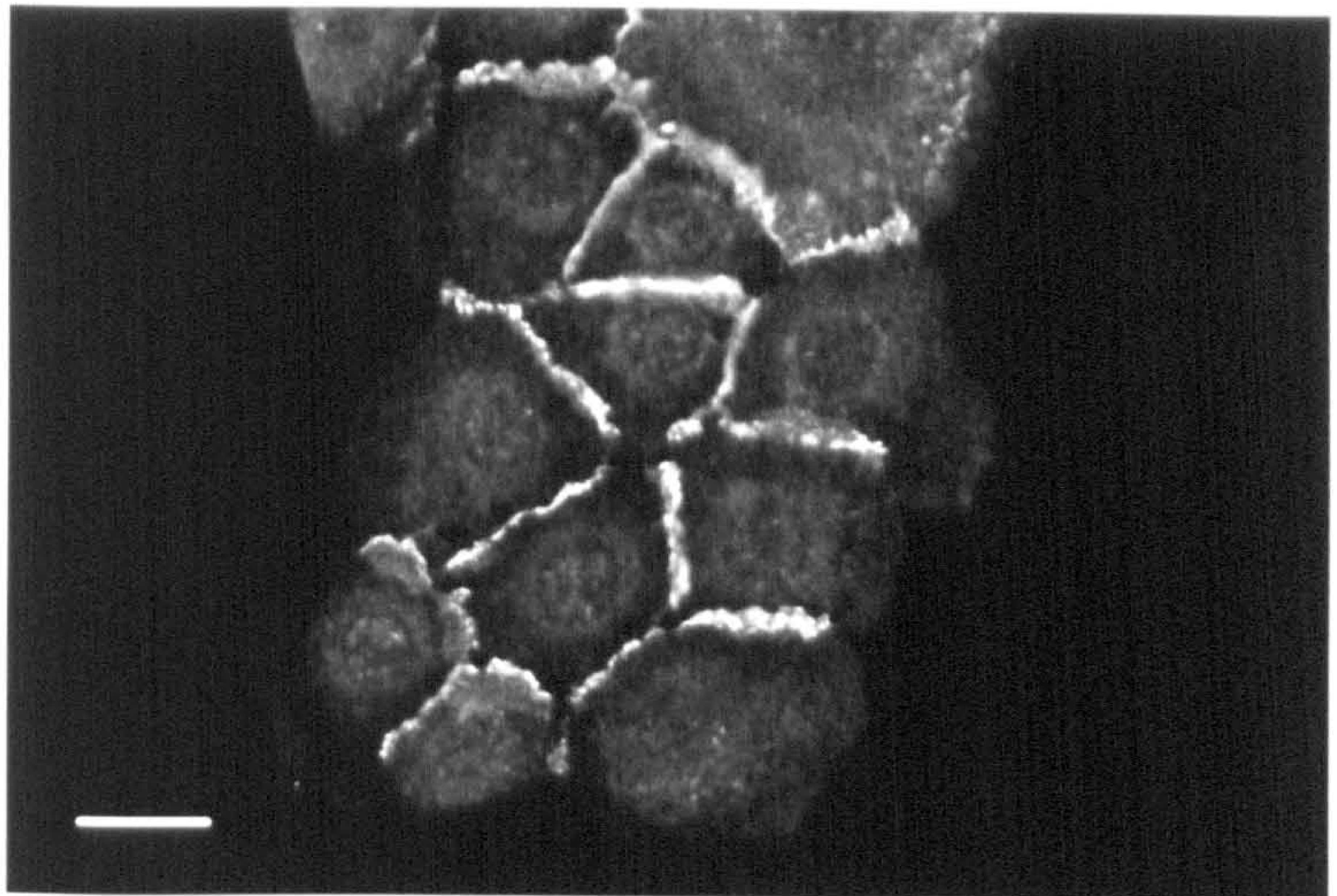
Confocal immunofluorescence micrographs of adherent keratinocytes stained for $p120^{ctn}$ after maintaining the cells in 0.03mM extracellular Ca^{++} (*a*) or after incubation in 1mM extracellular Ca^{++} for 4 hours (*b*) and 24 hours (*c*). The antiserum used was anti-$p120^{ctn}$, clone #98 diluted 1:100 visualised with FITC-conjugated anti-mouse IgG diluted 1:100. Scale bars represent 25μm.

High extracellular calcium induces p120^{ctn} relocalisation

a. low extracellular calcium



b. high extracellular calcium, 4 hours



c. high extracellular calcium, 24 hours

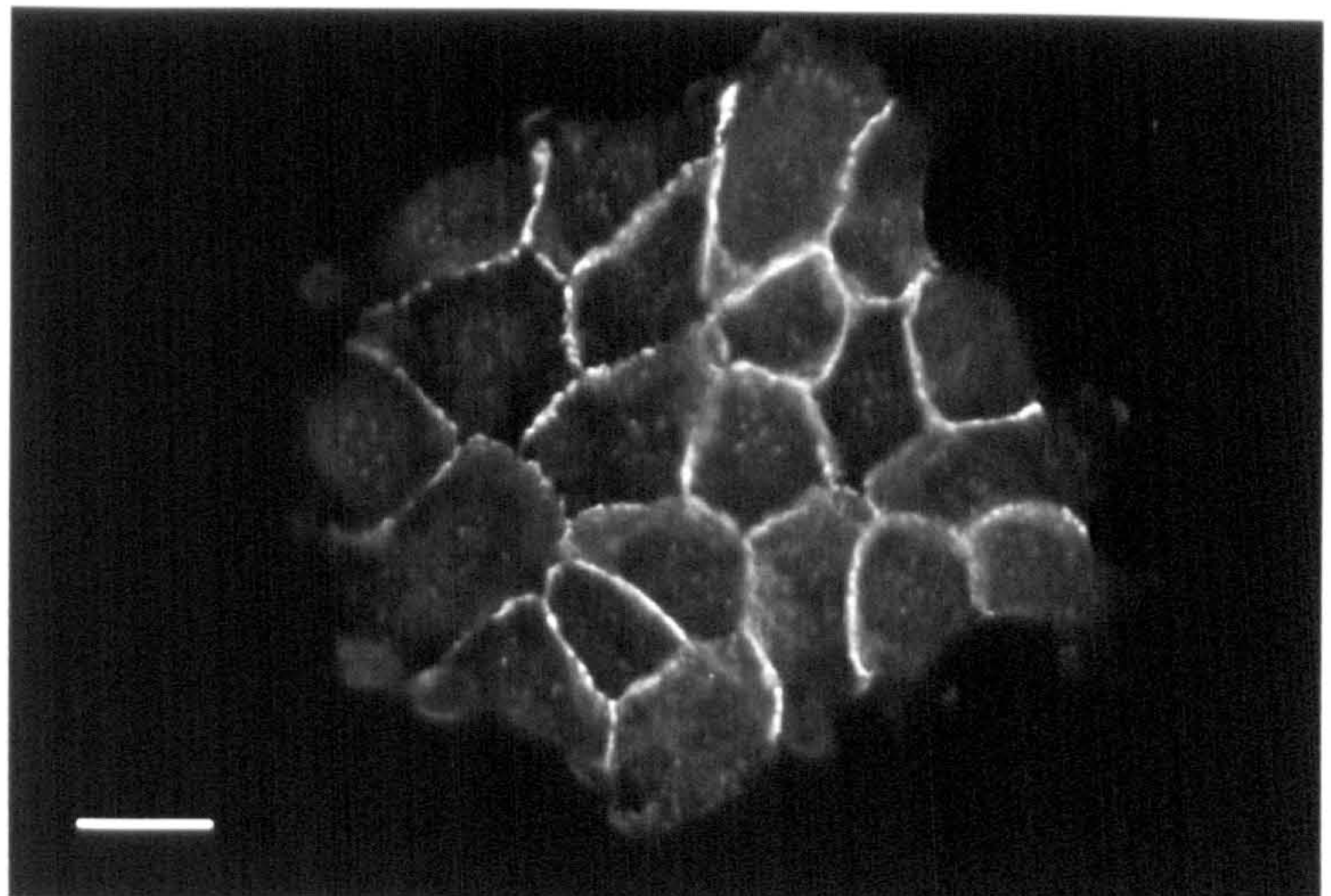
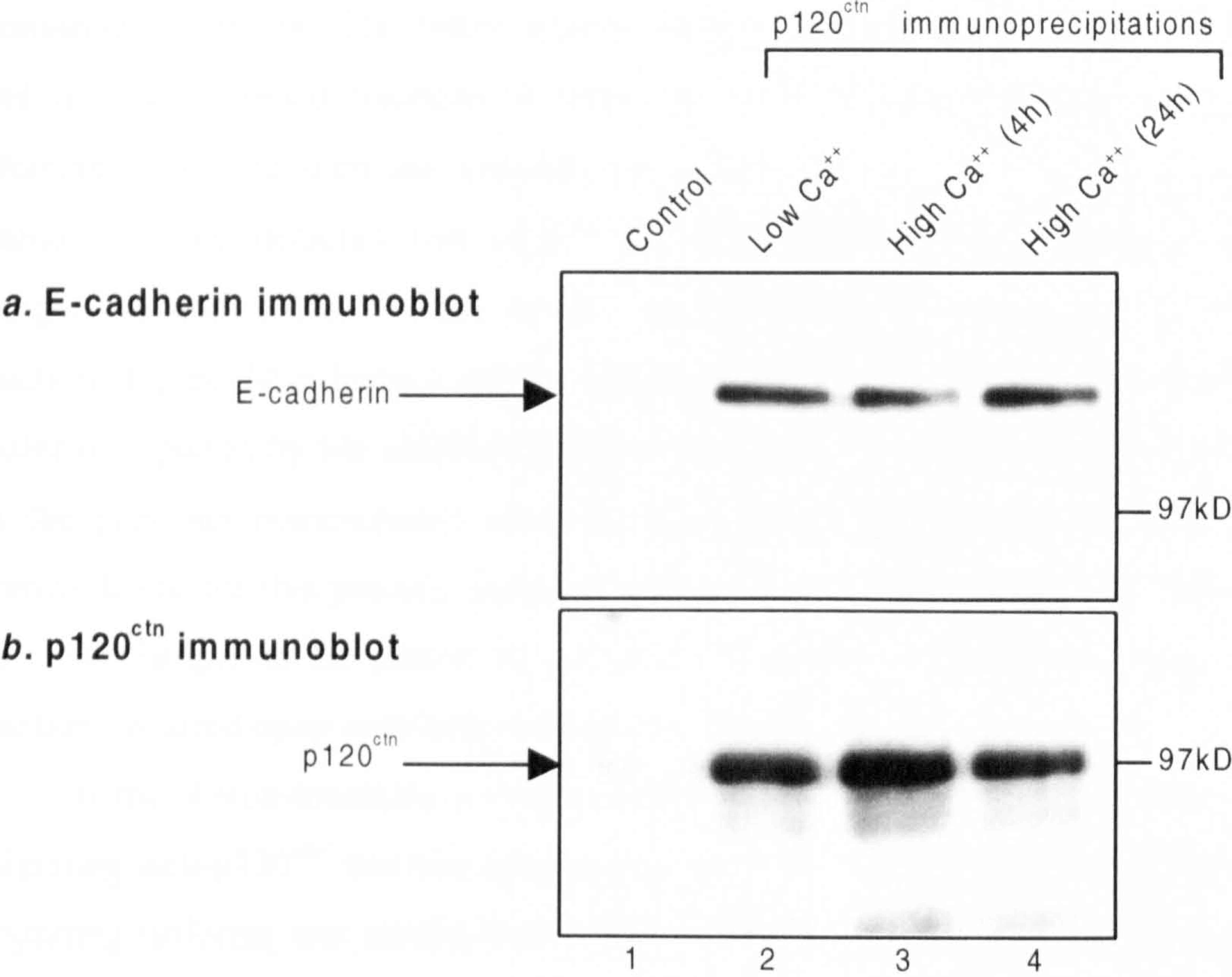


Figure 39

Figure 39
E-cadherin is co-immunoprecipitated by $p120^{ctn}$ antiserum

$p120^{ctn}$ immunoprecipitates immunoblotted for E-cadherin (*a*) and $p120^{ctn}$ (*b*). The $p120^{ctn}$ immunoprecipitations were performed on keratinocytes maintained in 0.03mM extracellular Ca^{++} and after 4 or 24 hours growth in 1mM extracellular Ca^{++}. Control immunoprecipitations (lane 1) were performed using normal mouse IgG. The antiserum used for immunoprecipitating was anti-$p120^{ctn}$ mAb clone #98 and the control antiserum was normal mouse IgG. The antisera used for immunoblotting were anti-E-cadherin mAb clone #36, diluted 1:2000 and anti-$p120^{ctn}$ mAb clone #98 diluted 1:1000. All immunoprecipitations were washed extensively in RIPA buffer.

E-cadherin is co-immunoprecipitated by p120^{ctn} antiserum



indicating that these two proteins may re-localise together in response to high extracellular Ca^{++}.

C. High extracellular calcium causes decreased $p120^{ctn}$ tyrosine phosphorylation

If the Src-family kinases co-localised with $p120^{ctn}$ in response to high extracellular Ca^{++} are catalytically active, then the amount of tyrosine phosphorylated $p120^{ctn}$ might increase if there are no compensatory changes in junctional tyrosine phosphatase activity. Figure 40 shows $p120^{ctn}$ immunoprecipitates immunoblotted for $p120^{ctn}$ (*a*) or phosphotyrosine (*b*). Keratinocytes were either maintained in 0.03mM extracellular Ca^{++} (lanes 1 and 3) or switched to 1mM extracellular Ca^{++} for 24 hours (lanes 2 and 4), before fractionation into the Triton-soluble and insoluble proteins (section 5.5) prior to immunoprecipitation. The Triton-soluble fraction is thought to contain mainly cytosolic and membrane-bound fractions whereas the Triton-insoluble fraction is enriched in proteins associated with the cytoskeleton (Hirano *et al.,* 1987; Hinck *et al.,* 1994). Immunoblotting detected two $p120^{ctn}$ isoforms migrating with apparent molecular weights around 100kD in the $p120^{ctn}$ immunoprecipitates from the Triton-soluble fraction (Figure 40 *a*, lanes 1 and 2). These are likely to correspond to the 'epithelial' isoforms reported by Mo and Reynolds (1996) which were named CAS2A and CAS2B in the previous nomenclature used for this protein. In accordance with the new nomenclature for this protein, these are labelled as $p120^{ctn}$3A and $p120^{ctn}$3B in Figure 40. No changes in the pattern of anti-$p120^{ctn}$ reactive proteins in the Triton-soluble fraction occurred upon switching the cells to HKGM.

In the Triton-insoluble extracts (Figure 40 *a,* lanes 3 and 4), a third, slower-migrating anti-$p120^{ctn}$ reactive species was present. The abundance of the 2 faster-migrating isoforms was similar in the Triton-soluble and insoluble extracts under both low Ca^{++} and high Ca^{++} growth conditions. However, the slower-migrating species was less abundant in cells switched to HKGM for 24 hours (lane 4, panel *a*). When this blot was probed using an anti-phosphotyrosine antiserum (panel *b*) it was apparent that the slower-migrating protein identified by the anti-$p120^{ctn}$ antiserum co-migrated with the largest of the tyrosine-phosphorylated proteins seen in lanes 3 and 4 of panel *b*. These data suggest that the largest protein identified by the $p120^{ctn}$ antiserum is tyrosine phosphorylated $p120^{ctn}$3A or $p120^{ctn}$3B. No tyrosine phosphorylated proteins could be detected in the $p120^{ctn}$ immunoprecipitates prepared from the Triton-soluble proteins.

The two other tyrosine phosphorylated proteins also present in the immunoprecipitations prepared from the Triton-insoluble proteins were not identified.

Thus, tyrosine phosphorylated p120^{ctn} was exclusively found in the Triton-insoluble fraction and became less abundant or showed reduced tyrosine phosphorylation during the time when cell adhesions were forming in response to high extracellular Ca^{++}. This is consistent with reports that elevated p120^{ctn} phosphorylation correlates with adherens junction disassembly (Papkoff, 1997; Kinch *et al.,* 1995). Taken together, these data indicate that tyrosine phosphorylated p120^{ctn} is exclusively associated with the cytoskeleton in keratinocytes but probably does not associate with E-cadherin. Furthermore, these experiments do not support the hypothesis that the Src kinases are activated at the adherens junctions during the formation of cell-cell adhesions.

9.3 Inhibiting Src-family kinase activity alters keratinocyte behaviour

The observations described in section 9.2 suggest that c-Src and the related Src kinases Fyn and c-Yes may play a role at keratinocyte adherens junctions although they may not be catalytically active during adhesion assembly. One function of the Src-family kinases in epithelial cells may involve the control of adherens junction stability since the expression of constitutively active v(viral)-Src causes the weakening of these adhesions in MDCK cells (Takeda *et al.,* 1995). However, it is also possible that the kinases localised to cell-cell adhesions transduce signals which influence cellular behaviour from the adherens junction into the cell. To define more accurately the functions of these kinases in keratinocytes, cells were treated with a Src-family kinase inhibitor (PD162531) generously provided to us by Dr. A. Kraker at Parke-Davis Pharmaceuticals. The structure of this compound was not disclosed to us due to patenting restrictions. This section includes a brief characterisation of some of the properties of this inhibitor and a description of its effects on keratinocyte adhesion and differentiation *in vitro*.

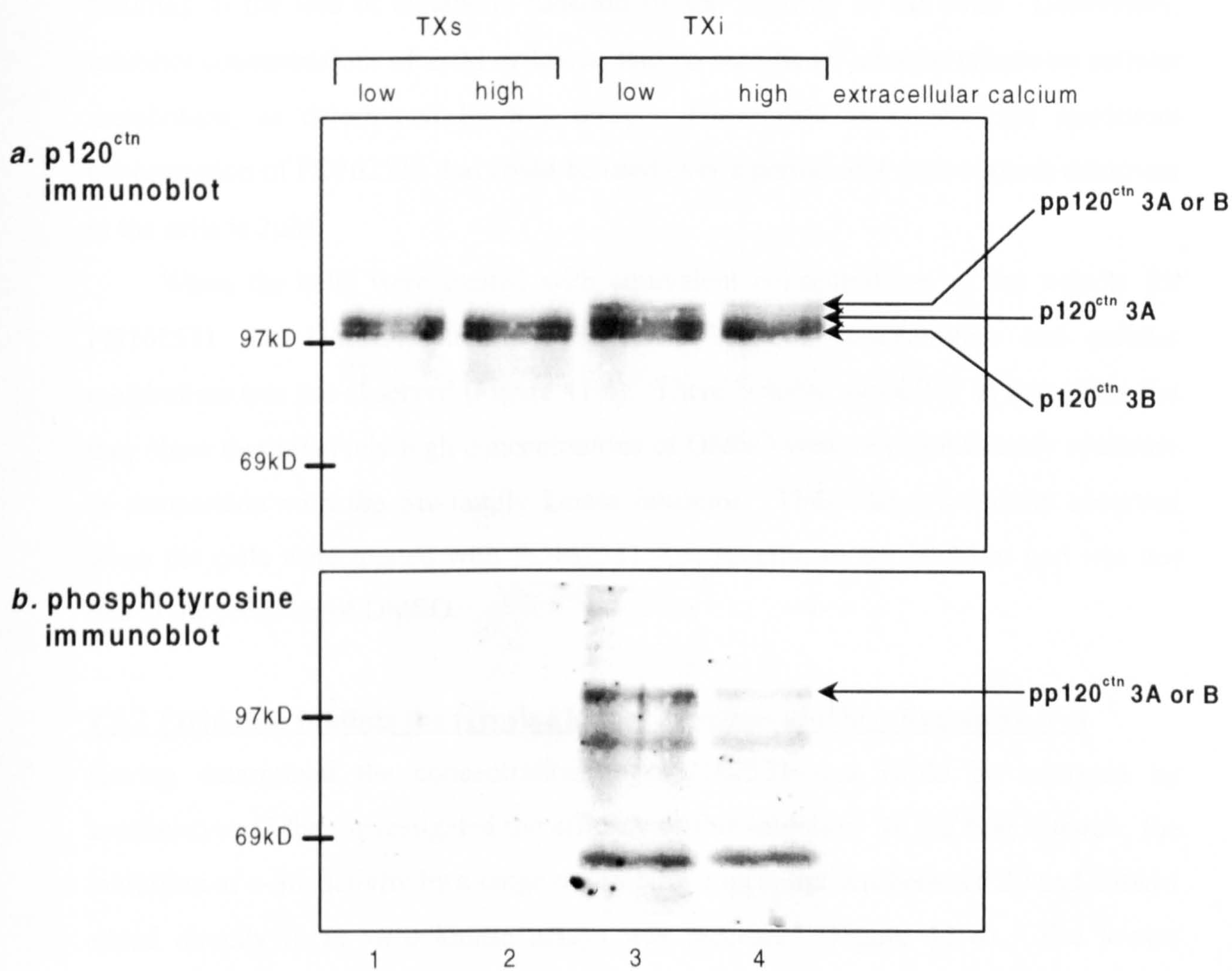
Figure 40

Figure 40

The effects of extracellular calcium on p120^{ctn} tyrosine phosphorylation

p120^{ctn} immunoprecipitates immunoblotted with anti-phosphotyrosine antiserum (panel *b*) and then with anti-p120^{ctn} (panel *a*) after removing the anti-phosphotyrosine antiserum (section 5.7.2). The lysates used in this experiment were prepared from adherent keratinocytes maintained in 0.03mM Ca^{++} or after 24 hours' incubation in 1mM extracellular Ca^{++}. Both Triton-soluble (TXs) and Triton-insoluble (TXi) extracts were used for the immunoprecipitations. The antisera used for immunoblotting were anti-p120^{ctn}, clone #98 diluted 1:1000 and anti-phosphotyrosine mAb PY20 diluted 1:1000.

The effects of extracellular calcium on $p120^{ctn}$ tyrosine phosphorylation



9.3.1 High concentrations of PD162531 are cytotoxic

To determine the concentrations of Src-kinase inhibitor which could be tolerated by human keratinocytes, the Promega CellTiter 96 assay was used to measure cell viability after 4 days incubation in inhibitor or an equivalent concentration of vehicle (DMSO, Figure 41). For these experiments, the cells were grown in 96-well plates in 0.03mM extracellular Ca^{++} medium for the duration of the experiment and the culture medium was replaced daily with fresh medium and inhibitor. Figure 41 *a* shows that inhibitor concentrations exceeding 2μM were cytotoxic with concentrations of 10μM or higher resulting in the loss of metabolic function by the majority of the cells. Conversely, inhibitor concentrations of 2μM or lower, had no significant adverse effects on cellular metabolism, as determined by this assay. These data show that the maximum concentration of PD162531 that could be used over a period of 4 days without detriment to the cells is 2μM.

When the cells were treated with equivalent concentrations of the vehicle for PD162531 (i.e. DMSO), such a relationship between concentration and cellular metabolism was not observed (Figure 41 *b*). There is some variability in these data but they show that relatively high concentrations of DMSO were not significantly cytotoxic in comparison with the Src-family kinase inhibitor. Thus, the cytotoxicity observed when the cells were treated with PD162531 was specific to the inhibitor and was not due to the presence of DMSO.

9.3.2 PD162531 inhibits the tyrosine kinase activities of c-Src, Fyn and c-Yes

Having determined the concentrations of PD162531 that could be tolerated by keratinocytes, I then investigated the efficacy of this inhibitor. In the first instance, the inhibition of c-Src activity by a range of inhibitor concentrations between 20 and 250nM added directly to *in vitro* kinase assays was measured (Figure 42 *a*). The lowest concentration of inhibitor tested had no measurable effect on c-Src activity but increasing the inhibitor concentration caused a corresponding decrease in c-Src kinase activity.

Next, a single inhibitor concentration (100nM) was tested for the inhibition of Fyn and c-Yes in the same way as described for c-Src (Figure 42 *b*). In this experiment, the activity of c-Src was again reduced by the inhibitor. Although the activities of Fyn and c-Yes as determined by this assay were considerably lower than that of c-Src, the

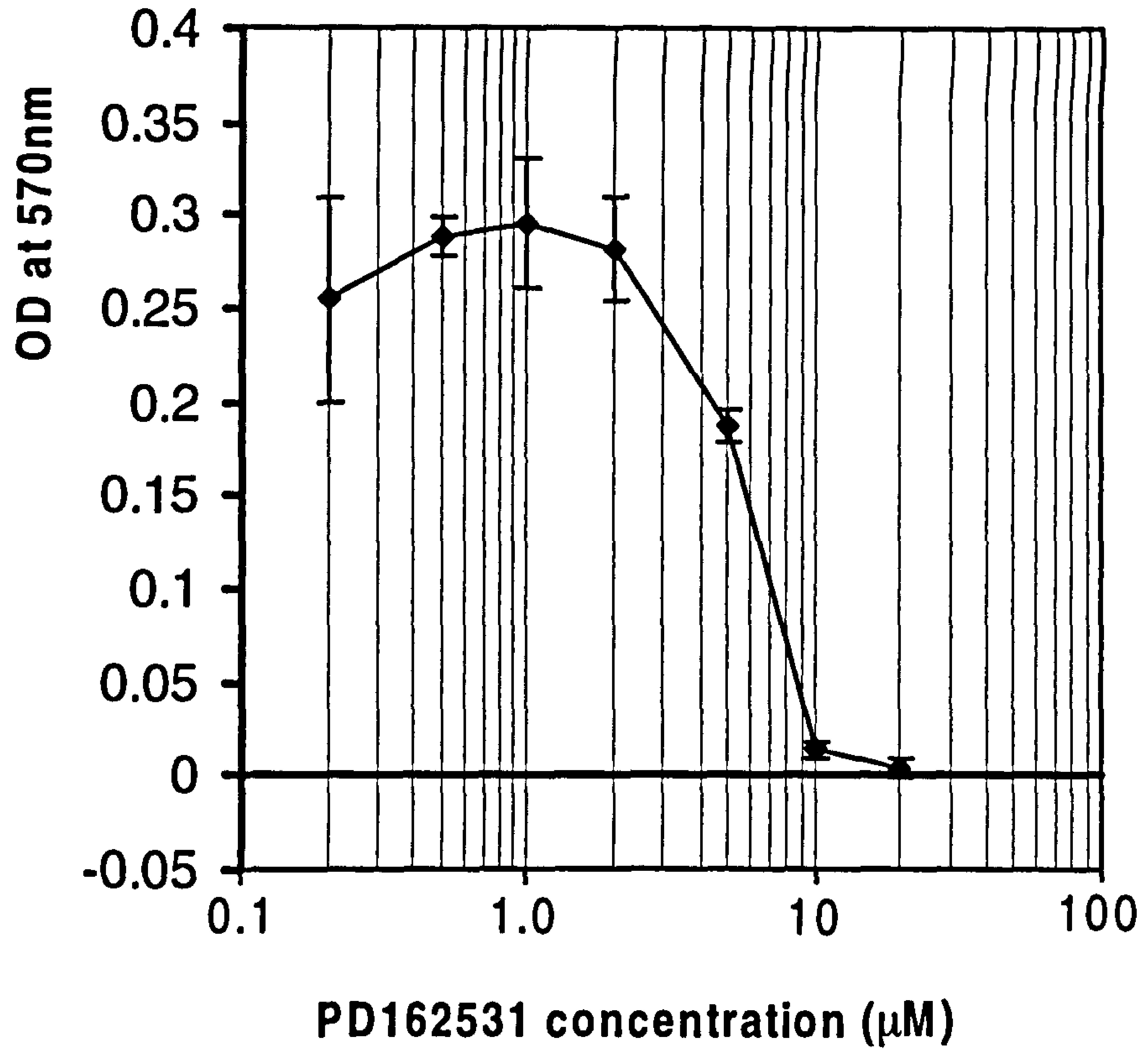
Figure 41

Figure 41
The cytotoxicity of PD162531

The effects of increasing PD162531 concentrations (*a*) or equivalent concentrations of the vehicle, DMSO (*b*) on cell viability assayed using the Promega CellTiter 96 kit after 4 days incubation in LKGM in the presence of PD162531 or DMSO. Cell viability is proportional to the optical density at 570nm where an absorbance of zero corresponds to no metabolising cells. Each data point represents the mean of three samples, +/- SEM.

The cytotoxicity of PD162531

a. **Cells treated with PD162531**



b. **Cells treated with DMSO**

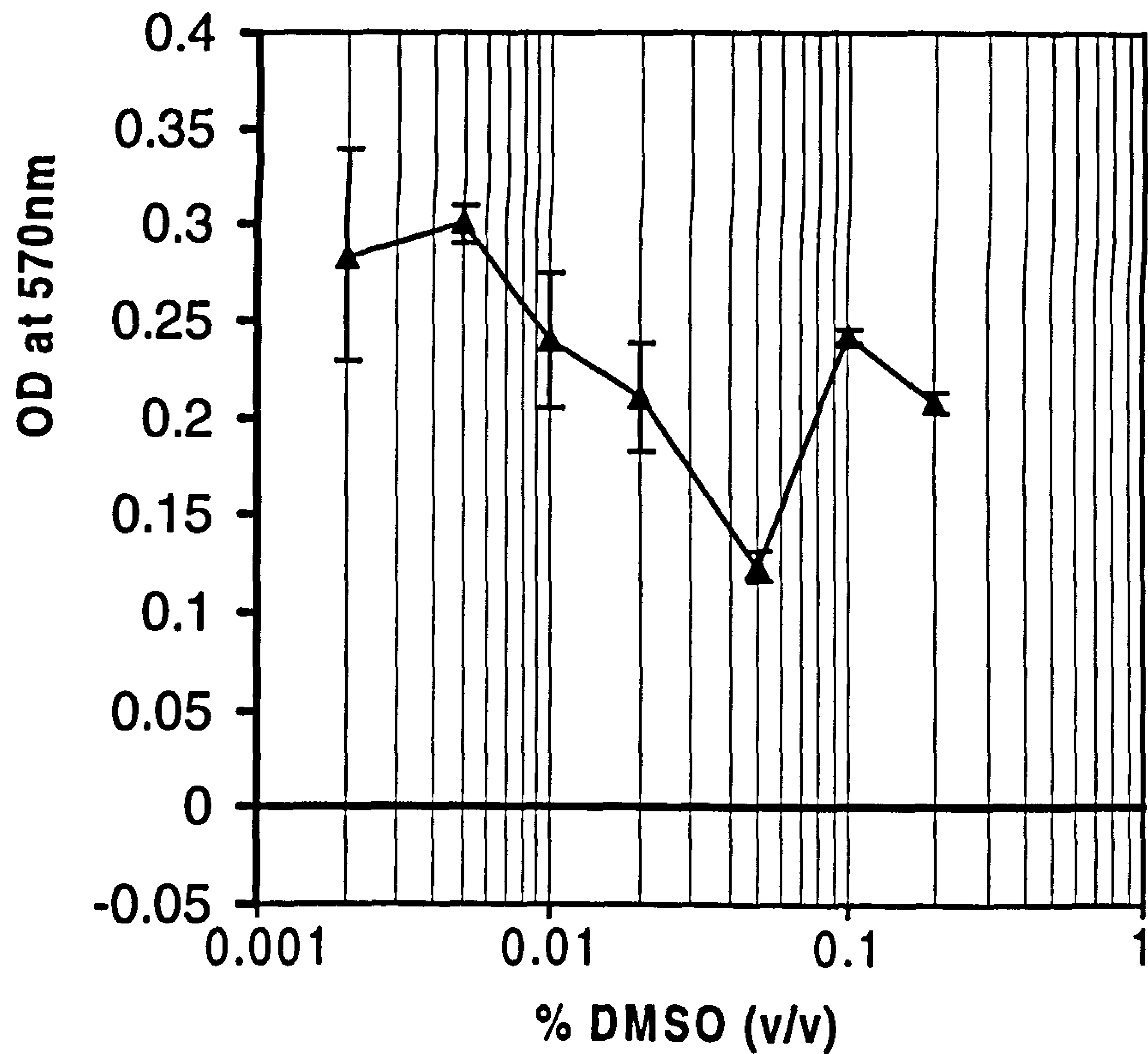


Figure 42

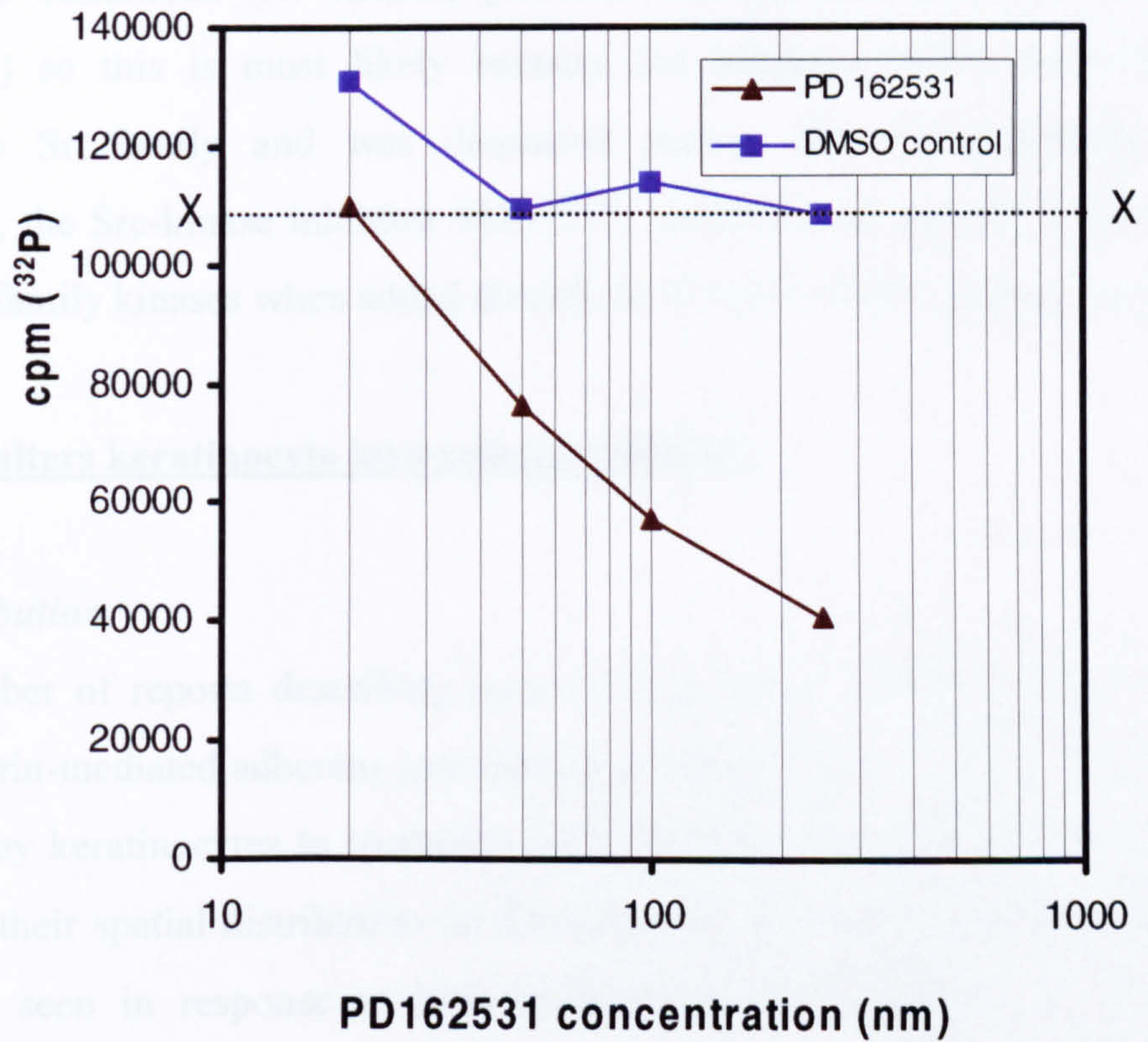
Figure 42

The effects of PD162531 on Src-family kinase activity

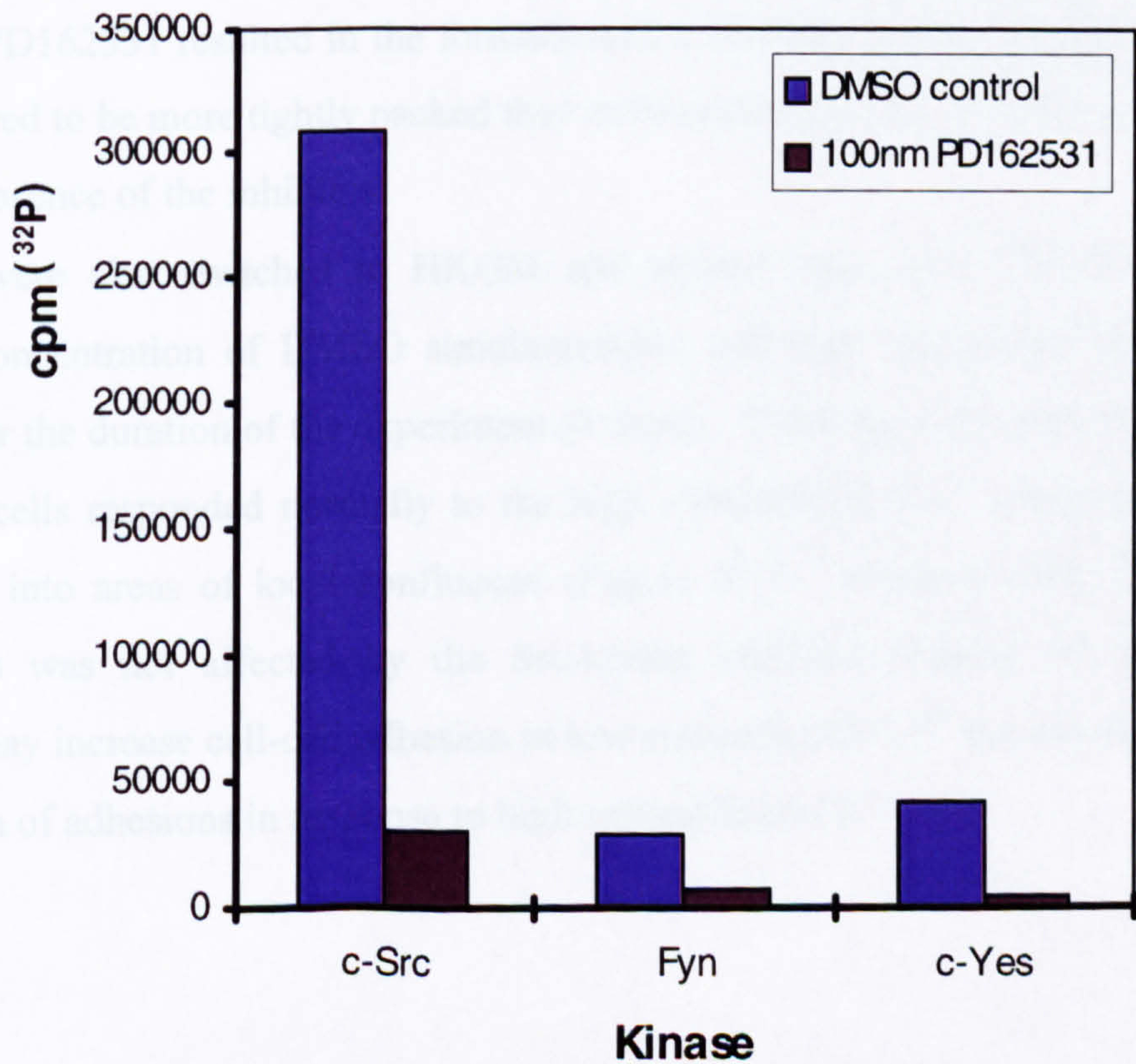
The effects of adding the Src-family kinase inhibitor PD162531 directly to *in vitro* kinase assays as (section 5.9). Chart *a* shows the effects of different inhibitor concentrations on c-Src activity whilst chart *b* shows the effects of 100nm inhibitor on c-Src, Fyn and c-Yes activity.

<u>**The effects of PD162531 on Src-family kinase activity**</u>

a. **c-Src kinase activity +/- PD162531**



b. **c-Src, Fyn and c-Yes activity +/- 100nm PD162531**



inhibitor also caused a marked decrease in their activity. However, using this type of kinase assay, we were unable to demonstrate that treating intact keratinocytes with PD162531 inhibited the Src kinases (not shown). The inhibitor is known to penetrate cells under these conditions (A. Kraker, personal communication to V. Brunton, Beatson Institute) so this is most likely because the inhibitor binds reversibly to members of the Src-family and was displaced during the immunoprecipitation procedure. Thus, the Src-kinase inhibitor PD162531 inhibited the activity of all three keratinocyte Src-family kinases when added directly to *in vitro* tyrosine kinase assays.

9.3.3 PD162531 alters keratinocyte intercellular adhesion

A. Cellular distribution

There are a number of reports describing how the Src-family kinases can affect the stability of cadherin-mediated adherens junctions (e.g. Takeda *et al.,* 1995). One of the responses made by keratinocytes to treatment with the Src-kinase inhibitor in LKGM was a change in their spatial distribution on the substrate in a manner similar, but not identical to that seen in response to high extracellular Ca^{++} (Figure 43). When keratinocytes were treated with PD162531 at a concentration of 2μM in LKGM, the distribution of the cells changed from the scattered morphology characteristic of low extracellular Ca^{++} (Figure 43 *a*), to a more ordered arrangement (Figure 43 *c*) which was visible within hours of the addition of the inhibitor. This redistribution of the cells in response to PD162531 resulted in the formation of a number of discrete groups of cells which appeared to be more tightly packed than cells maintained in 0.03mM extracellular Ca^{++} in the absence of the inhibitor.

Cells were also switched to HKGM and treated with 2μM PD162531 or an equivalent concentration of DMSO simultaneously and then maintained under these conditions for the duration of the experiment (4 days). When the cells were treated with DMSO, the cells responded normally to the high extracellular Ca^{++} concentration and redistributed into areas of local confluence (Figure 43 *b* - compare with Figure 13). This process was not affected by the Src-kinase inhibitor (Figure 43 *d*). Thus, PD162531 may increase cell-cell adhesion in low extracellular Ca^{++} but has no effect on the formation of adhesions in response to high extracellular Ca^{++}.

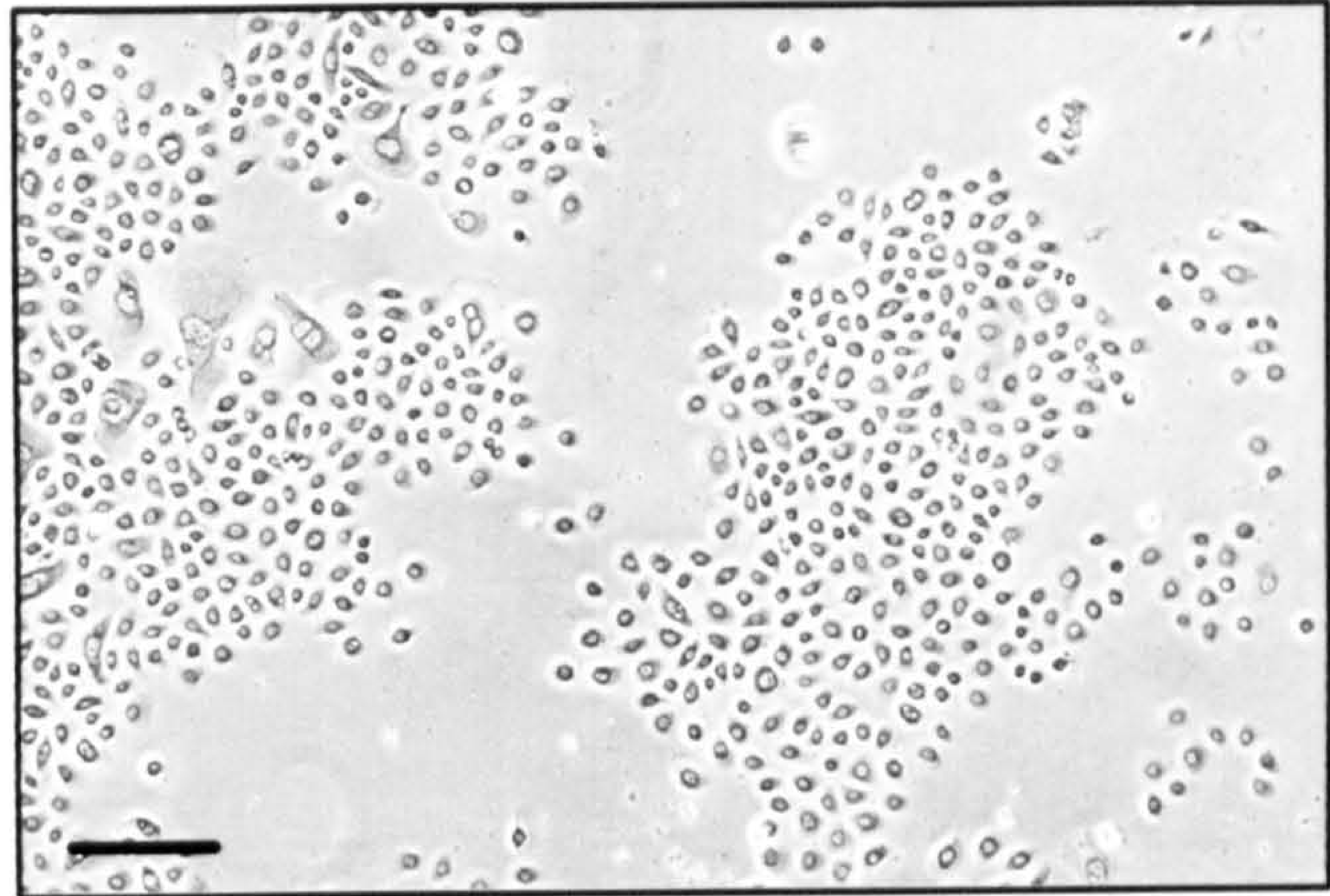
Figure 43

Figure 43
PD162531 alters keratinocyte spatial distribution

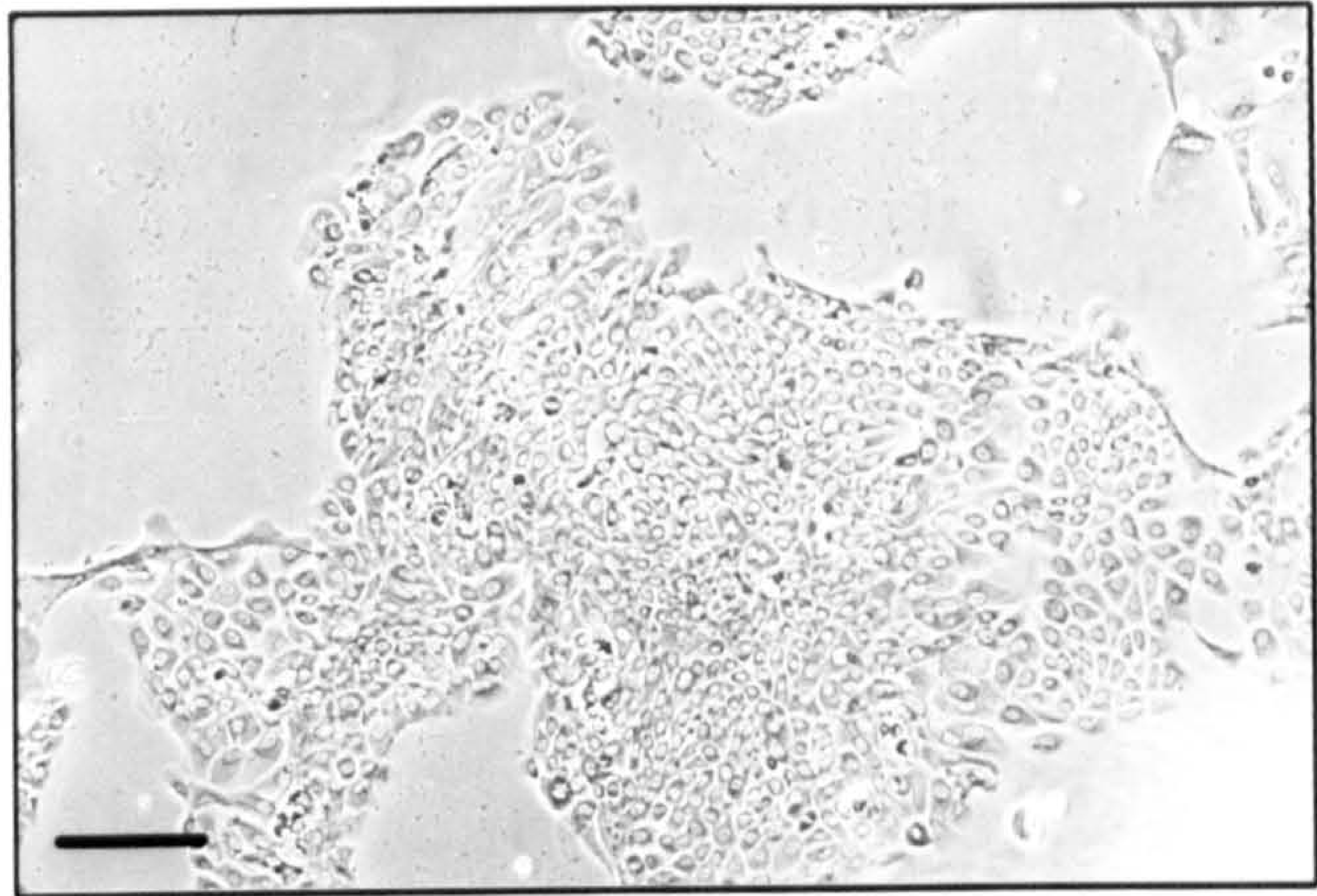
Phase-contrast photomicrographs of subconfluent adherent keratinocytes maintained in 0.03mM extracellular Ca^{++} (panels *a* and *c*) or switched to 1mM extracellular Ca^{++} for a duration of 4 days (panels *b* and *d*). The cells shown in panels *a* and *b* were incubated in 0.02% (v/v) DMSO and the cells shown in panels *c* and *d* were incubated in 2μM PD162531 for the 4 day duration of the experiment. The scale bars represent 200μm.

PD162531 treatment alters keratinocyte spatial distribution

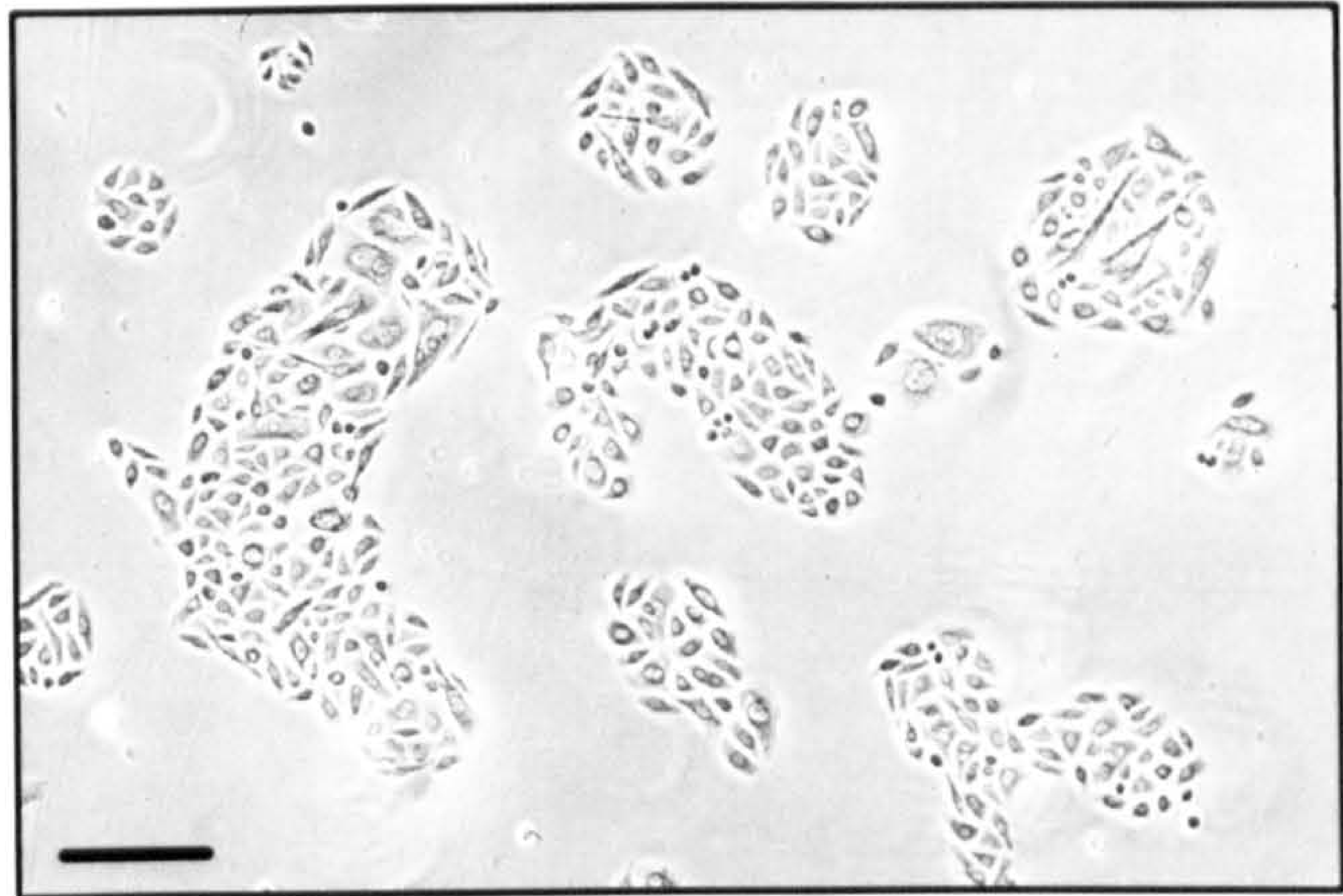
a. low extracellular calcium, DMSO



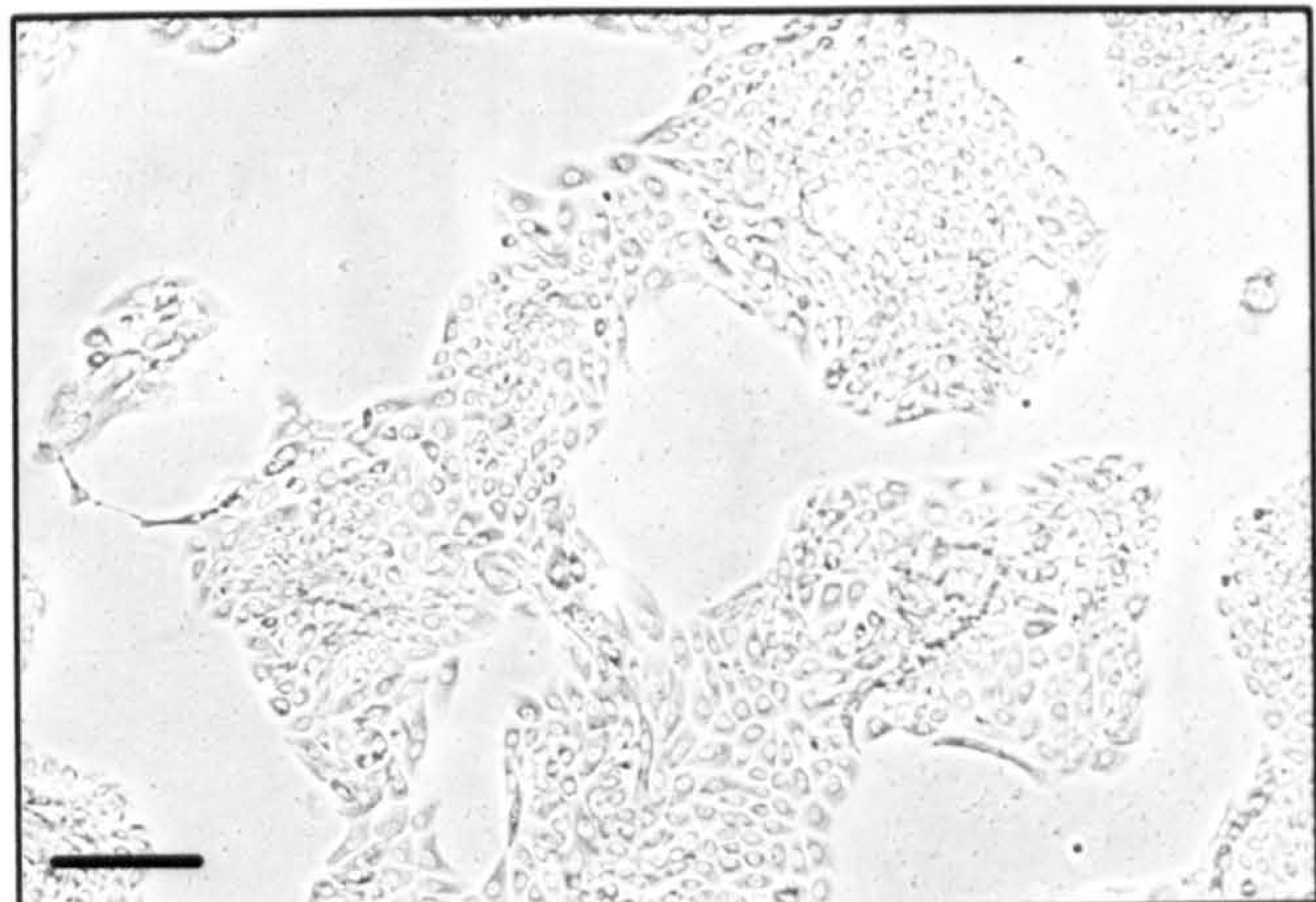
b. high extracellular calcium, DMSO



c. low extracellular calcium, PD162531



d. high extracellular calcium, PD162531



B. Intercellular adhesion

The data presented in section 9.3.3A suggested that one effect of the Src-family kinase inhibitor PD162531 may be to increase cell-cell adhesion in some way. To investigate this, subconfluent adherent keratinocytes were treated with the inhibitor (or DMSO for controls) in 0.03mM or 1mM extracellular Ca^{++} for a period of 24 hours. The cells were then fixed and stained for E-cadherin or the β_1-integrin subunit.

In low extracellular Ca^{++}, in the absence of PD162531, both E-cadherin and the β_1-integrin subunit were diffusely localised in the majority of the cells (Figures 44 *a* and 45 *a*). However, both proteins were weakly concentrated to the sporadic cell-cell contact areas and in addition, some punctate staining for β_1-integrin could be seen at the sites of attachment to the substrate. These micrographs show that DMSO had no significant effect on the subcellular distribution of the two adhesion proteins (compare with Figures 16 *a* and 21 *a*). Furthermore, when keratinocytes were incubated in HKGM for 24 hours in the presence of DMSO, the redistribution of E-cadherin and the β_1-integrin subunit to sites of cell-cell adhesion proceeded normally (Figures 44 *b* and 45 *b* - compare with Figures 16 *b* and 21 *b*).

Treating the cells with PD162531 in low extracellular Ca^{++} caused the cells to become more tightly packed (Figures 44 *c* and 45 *c*) and their morphology was similar, but not identical, to cells induced to aggregate by high extracellular Ca^{++} concentrations. In these closely apposed cells, E-cadherin was localised along the areas of cell-cell contact although this was weaker than was seen in response to high extracellular Ca^{++} (Figure 44 *c*). No significant cell-cell staining for the β_1-integrin subunit was seen in cells treated with PD162531 in LKGM (Figure 45 *c*). Treating the cells with PD162531 at the same time as elevating the extracellular Ca^{++} concentration to 1mM had no effect on the redistribution of the cells in response to Ca^{++} (Figure 43 *d*). Similarly, the relocalisation of both E-cadherin (Figure 44 *d*) and the β_1-integrin subunit (Figure 45 *d*) were unaffected. Thus, the formation of cell-cell adhesions in response to high extracellular Ca^{++} is not affected by the presence of PD162531. The intercellular adhesions formed in low extracellular Ca^{++} in the presence of the inhibitor may involve E-cadherin but probably not β_1-integrin.

Figure 44

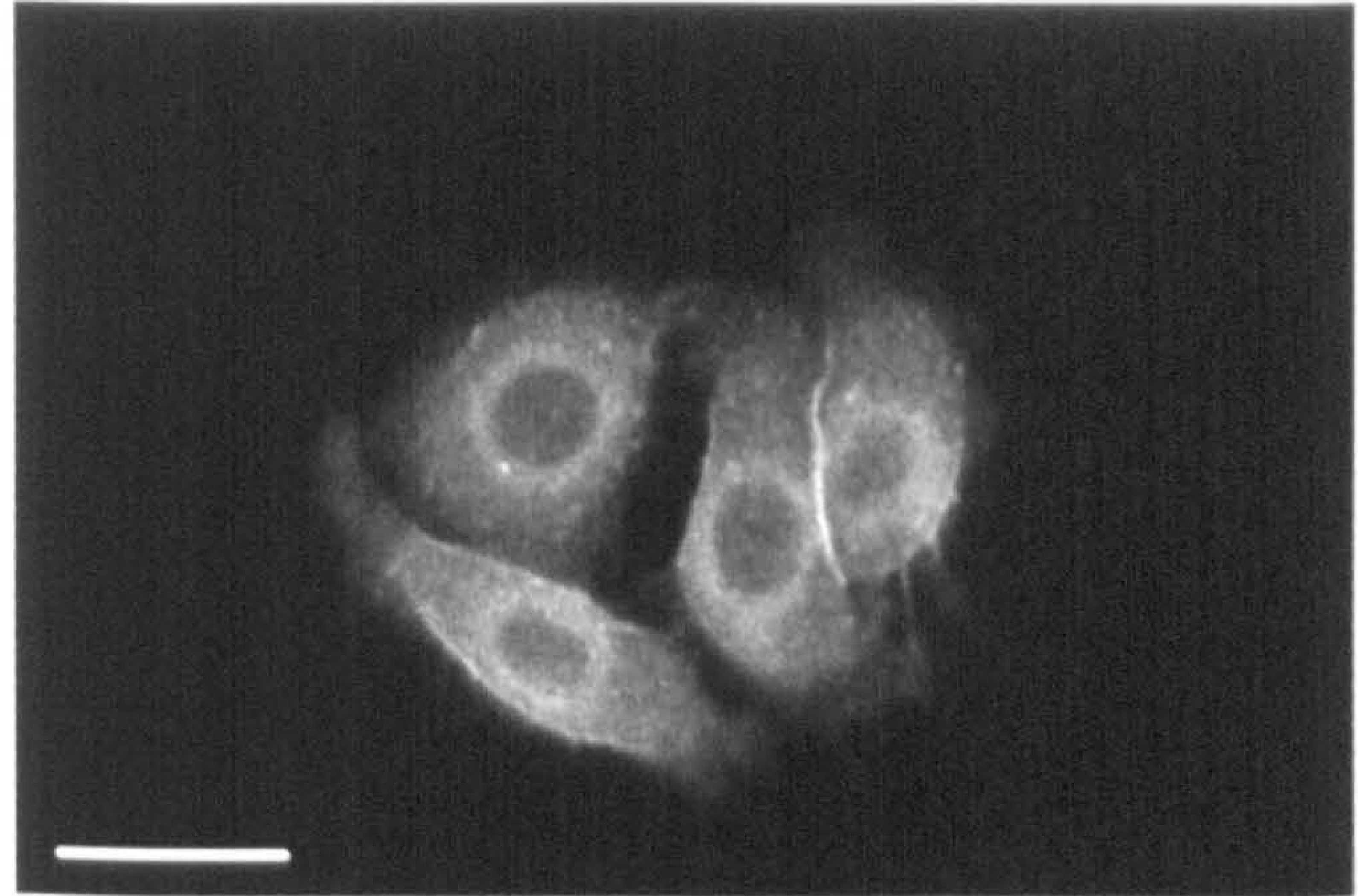
Figure 44

The effects of PD162531 on the subcellular localisation of E-cadherin

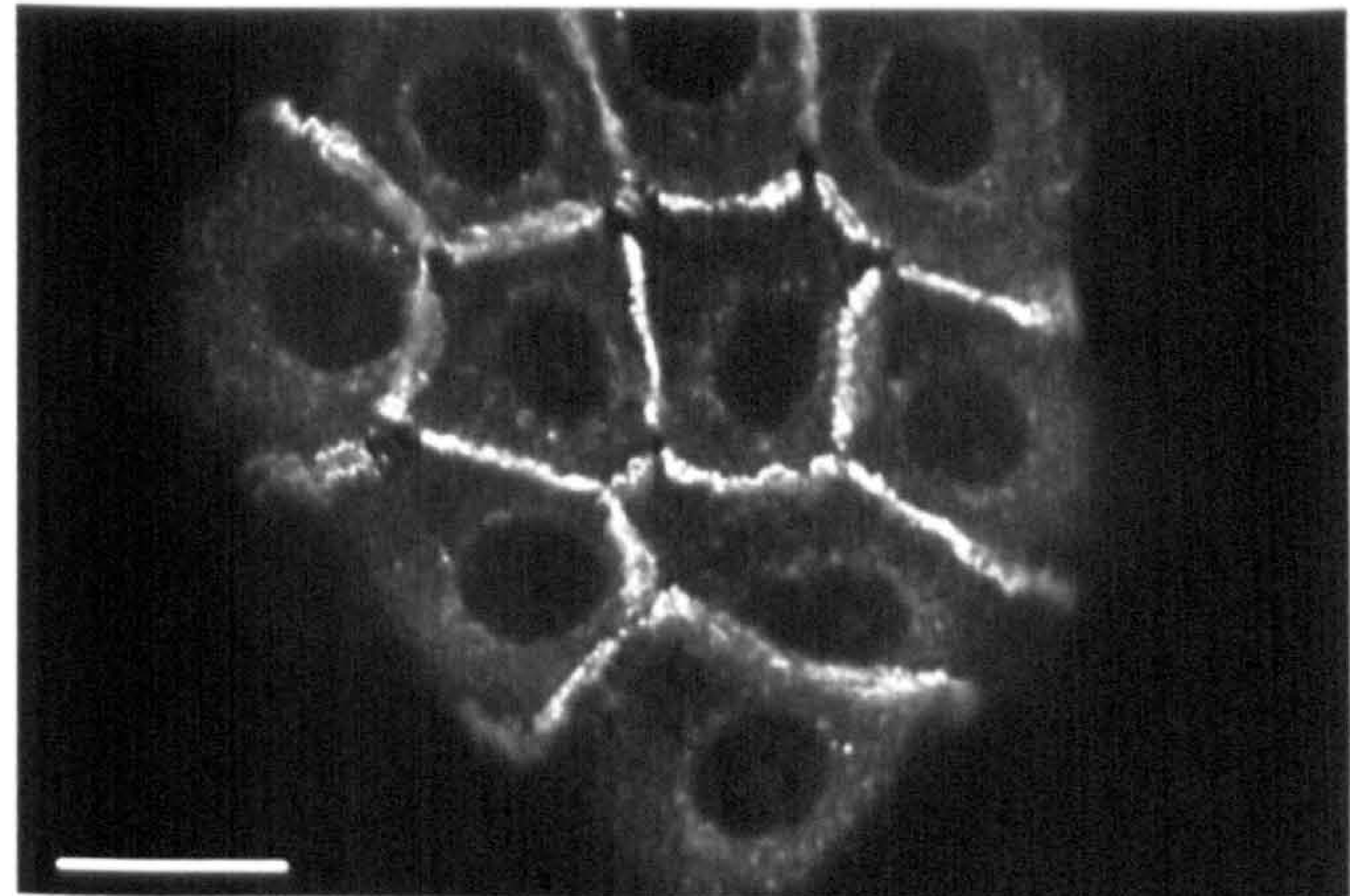
Confocal immunofluorescence micrographs of adherent keratinocytes stained for E-cadherin after maintenance in 0.03mM extracellular Ca^{++} (images *a* and *c*) and after 24 hours incubation in 1mM extracellular Ca^{++} (images *b* and *d*). The cells shown in images *a* and *b* were treated with 0.02% (v/v) DMSO for the 24 hour duration of the experiment whereas the cells depicted in images *c* and *d* were treated with 2μM PD162531. The antiserum used was anti-E-cadherin mAb clone #36 diluted 1:250. Scale bars represent 25μm.

The effects of PD162531 on the subcellular localisation of E-cadherin

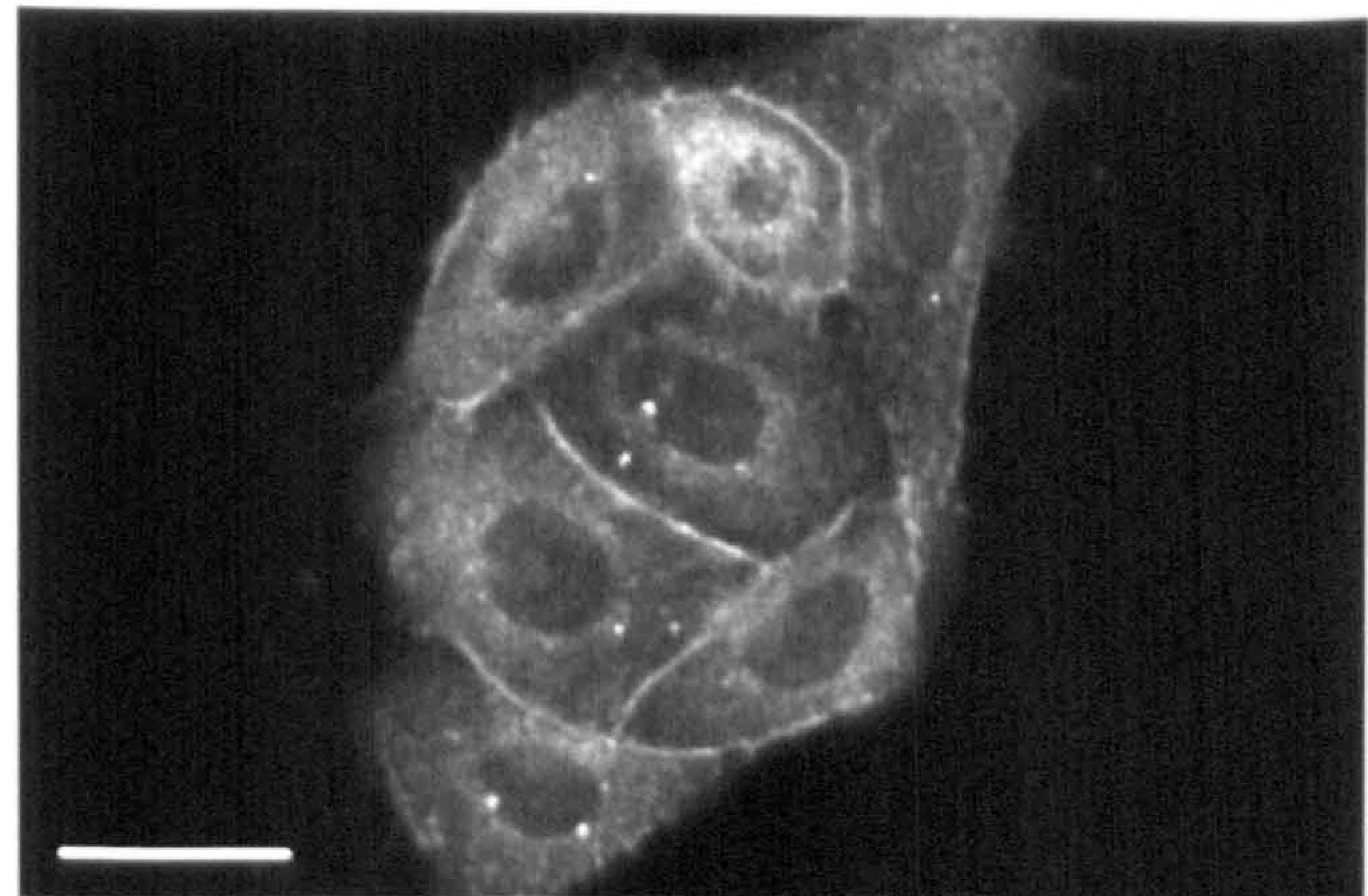
a. low extracellular calcium, DMSO



b. high extracellular calcium and DMSO for 24 hours



c. low extracellular calcium and PD162531 for 24 hours



d. high extracellular calcium and PD162531 for 24 hours

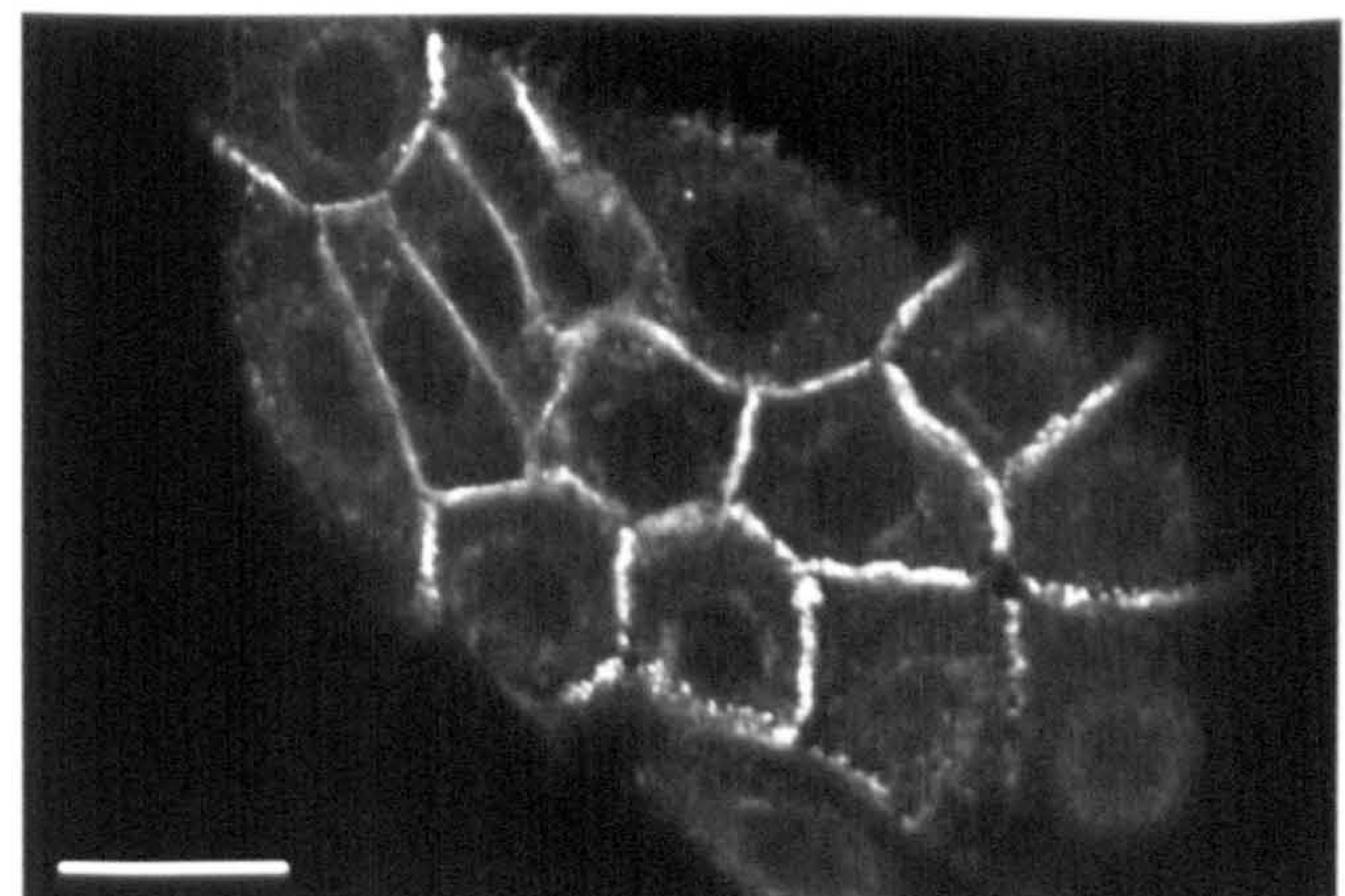


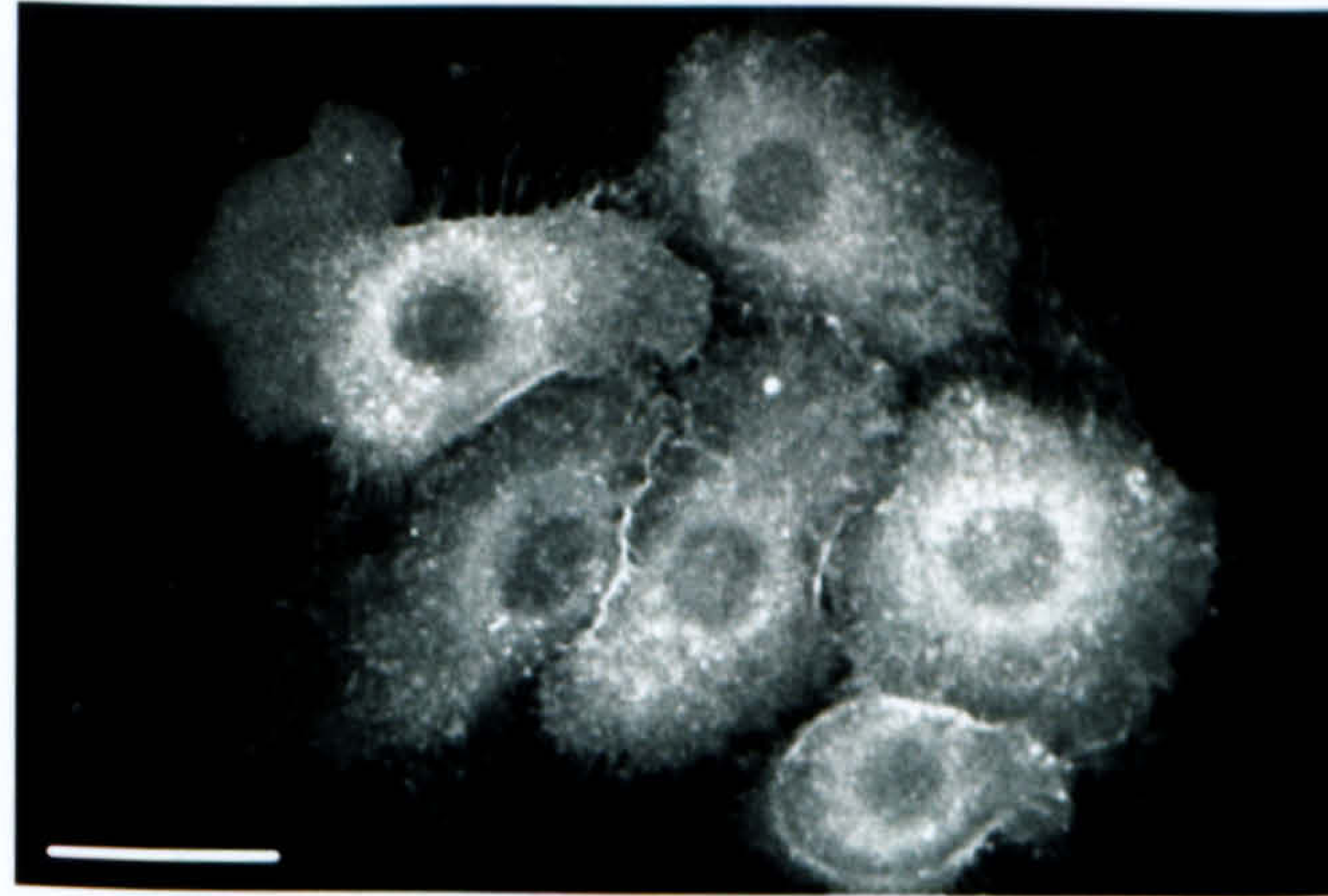
Figure 45

Figure 45
The effects of PD162531 on the subcellular localisation of β_1-integrin

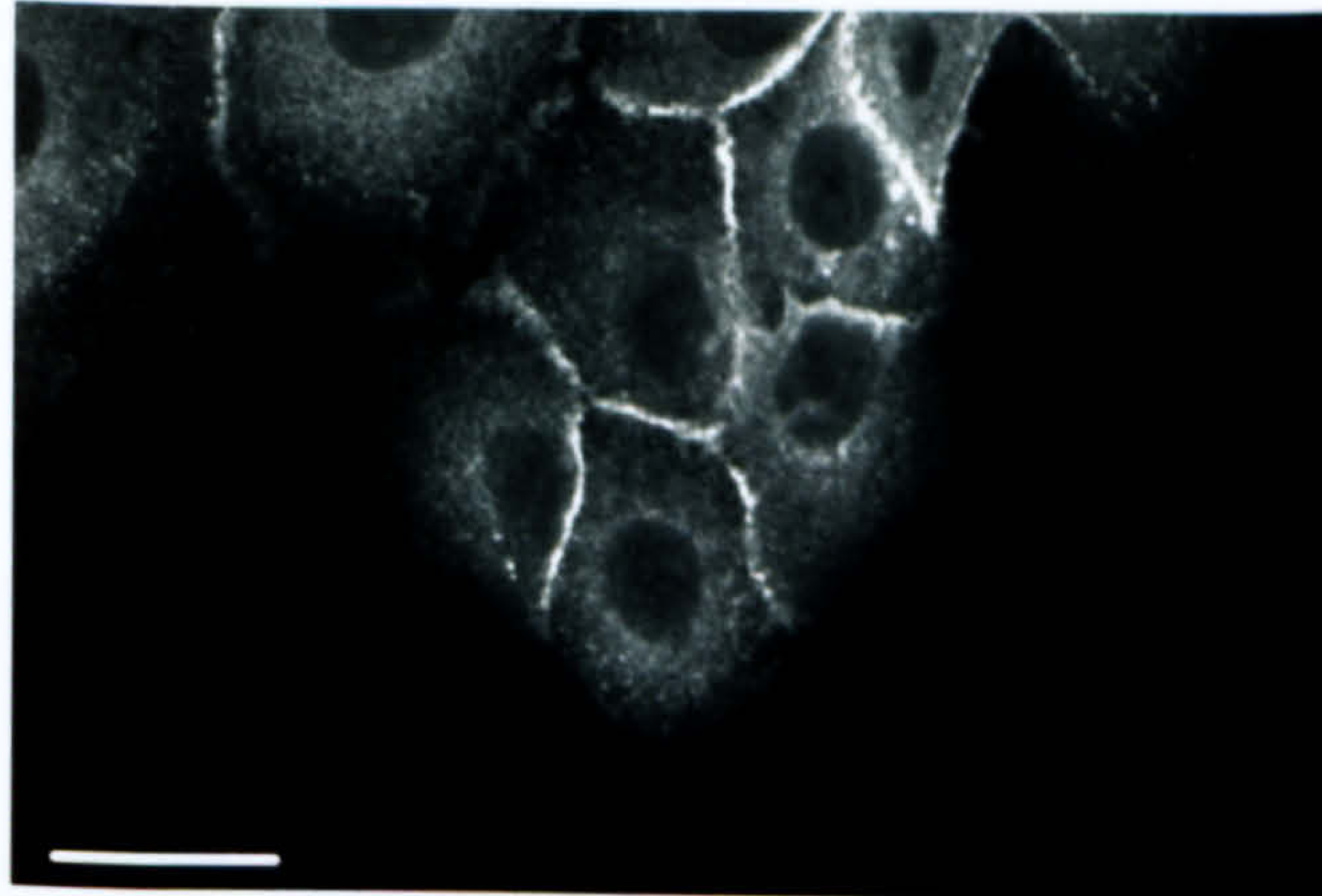
Confocal immunofluorescence micrographs of adherent keratinocytes stained for the β_1-integrin subunit after maintenance in 0.03mM extracellular Ca^{++} (images *a* and *c*) or after incubation in 1mM extracellular Ca^{++} for a period of 24 hours (images *b* and *d*). The cells depicted in images *a* and *b* were treated with 0.02% (v/v) DMSO and those shown in images *c* and *d* were treated with 2μM PD162531 for the 24 hour duration of the experiment. The antiserum used was anti-β_1-integrin mAb 13 diluted 1:100. The scale bars represent 25μm.

The effects of PD162531 on the subcellular localisation of β_1-integrin

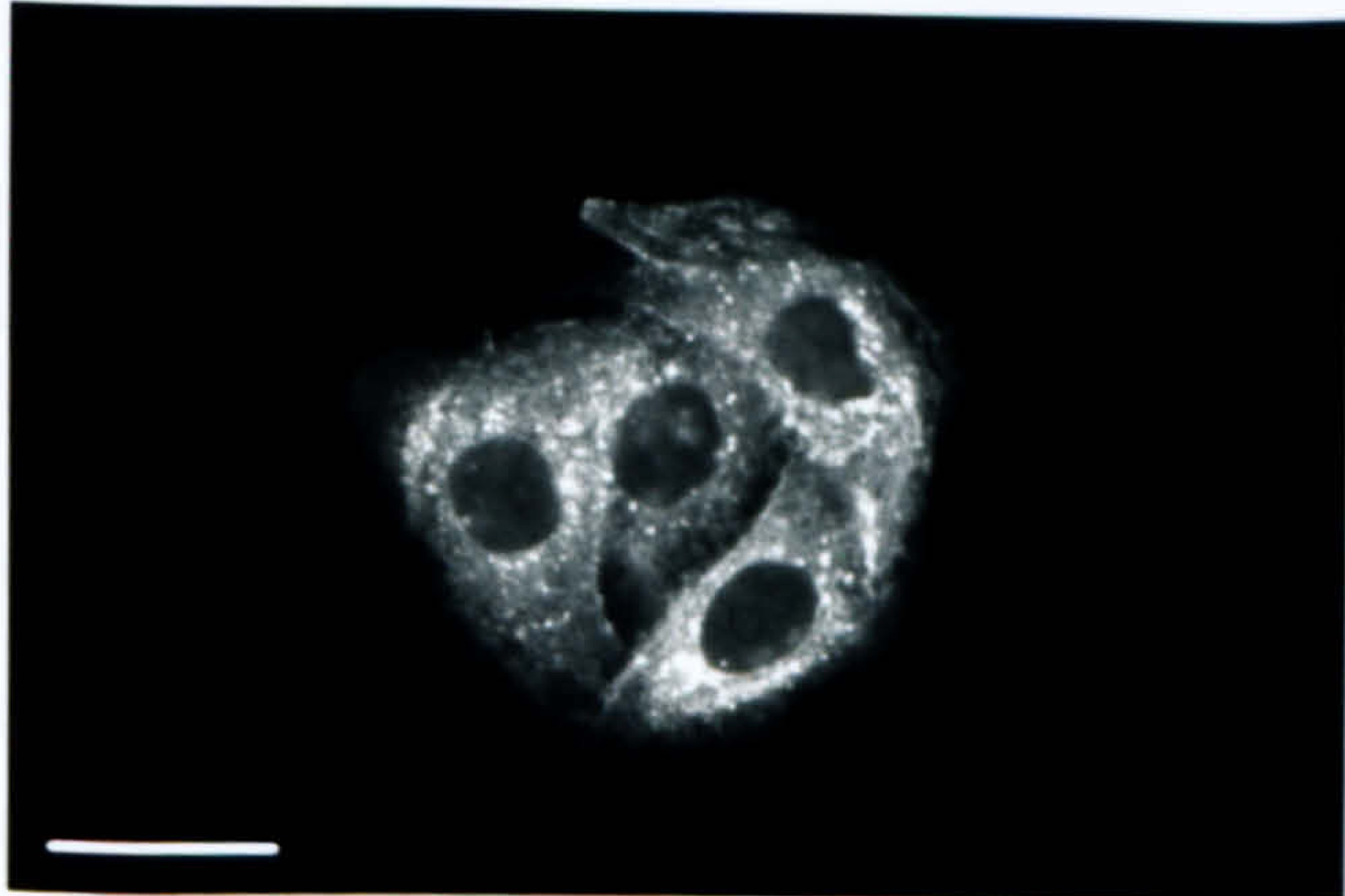
a. low extracellular calcium, DMSO



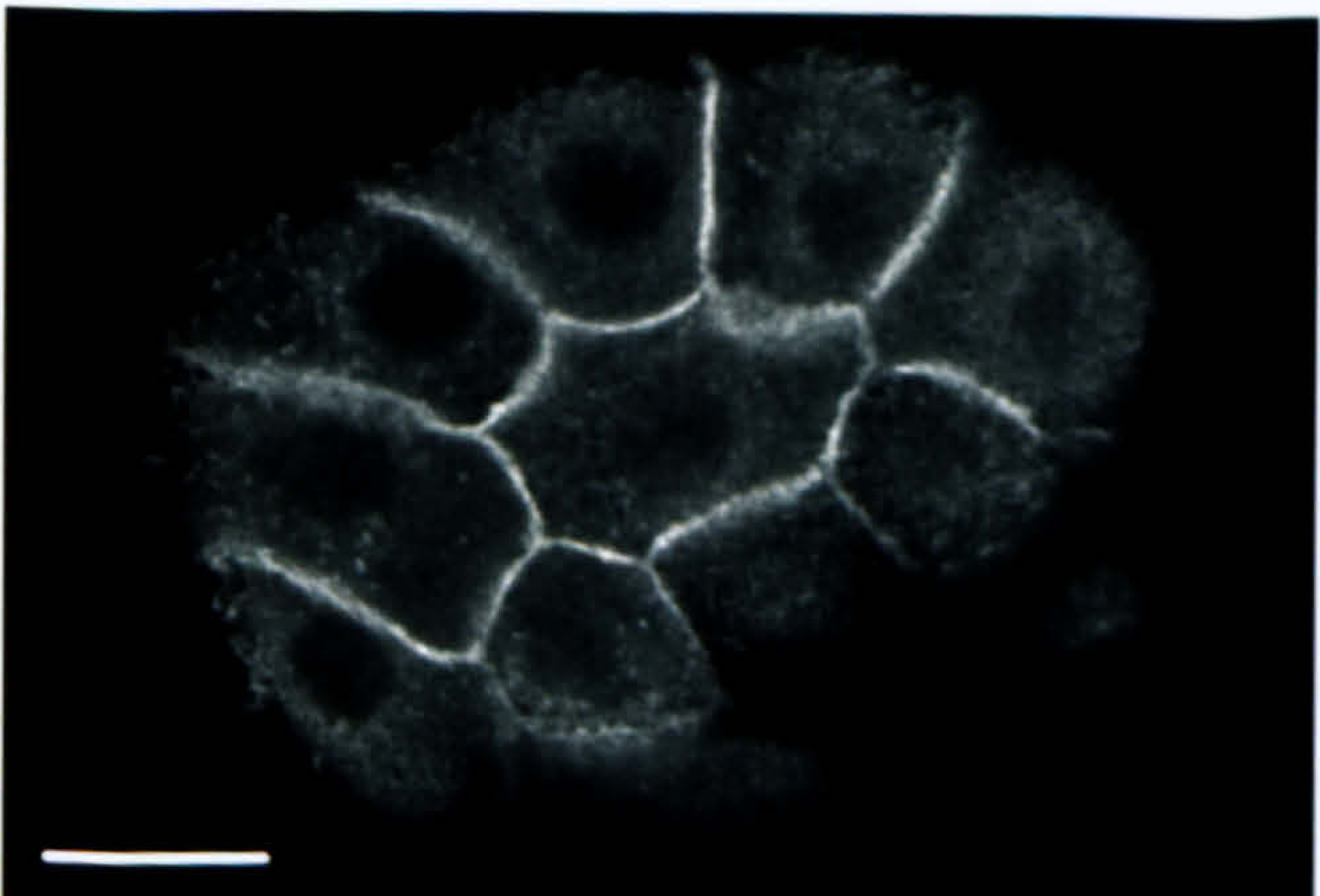
b. high extracellular calcium and DMSO for 24 hours



c. low extracellular calcium and PD162531 for 24 hours



d. high extracellular calcium and PD162531 for 24 hours



9.3.4 PD162531 does not affect keratinocyte differentiation

A number of reports have indicated that members of the Src-family kinases may play a role in the regulation of keratinocyte differentiation. In particular, keratinocytes derived from *fyn* -/- mice fail to differentiate normally in response to high extracellular Ca^{++} concentrations *in vitro* (Calautti *et al.,* 1995). These cells express some differentiation-specific proteins normally in response to this stimulus, but the expression of others such as filaggrin is delayed.

To investigate the effects of PD162531 on differentiation, adherent subconfluent keratinocytes were treated with 2μM PD162531 or an equivalent concentration of DMSO for up to 5 days before involucrin expression was determined by immunoblotting (Figure 46 *a* and *b*) and filaggrin expression by immunoperoxidase staining (Figure 47). In low extracellular Ca^{++}, the levels of involucrin were low (Figure 46 *a*) and were not significantly affected by the presence of DMSO or PD162531. Similarly, high extracellular Ca^{++} strongly induced the expression of involucrin and this was unaffected by the presence of the inhibitor. Thus, under these conditions, inhibiting members of the Src-family did not affect the Ca^{++}-induced expression of involucrin, nor did it affect involucrin expression in 0.03mM extracellular Ca^{++}.

When keratinocytes incubated in HKGM in the presence of 0.02% (v/v) DMSO for 5 days were stained for filaggrin, some cells were expressing this protein (Figure 47 *a* and 47 *b*). As was previously described in section 6.2.1, the proportion of cells expressing filaggrin was modest in comparison with the proportion which express involucrin and transglutaminase under similar conditions. When the cells were treated with PD162531 and incubated in HKGM for 5 days, the proportion of cells expressing filaggrin was similar to the cultures treated with DMSO (Figure 47 *c* and *d*). Thus, treating keratinocytes with 2μM PD162531 did not interfere with the Ca^{++}-induced expression of filaggrin. Due to the difficulties inherent in attempting to distinguish single cells in these stratified groups of cells, it was not possible to quantitate the proportion of cells expressing filaggrin.

Figure 46

Figure 46
The effects of PD162531 on involucrin expression

Involucrin (*a* and *b*) and α-tubulin (*c*) immunoblots prepared using proteins extracted using CSK buffer from subconfluent adherent keratinocytes maintained in LKGM or HKGM in the presence of 0.02% (v/v) DMSO or 2μM PD162531 for 4 days. The antisera used were anti-involucrin mAb SY5 diluted 1:1000 and anti-α-tubulin mAb DM 1A diluted 1:10000.

The effects of PD162531 on involucrin expression

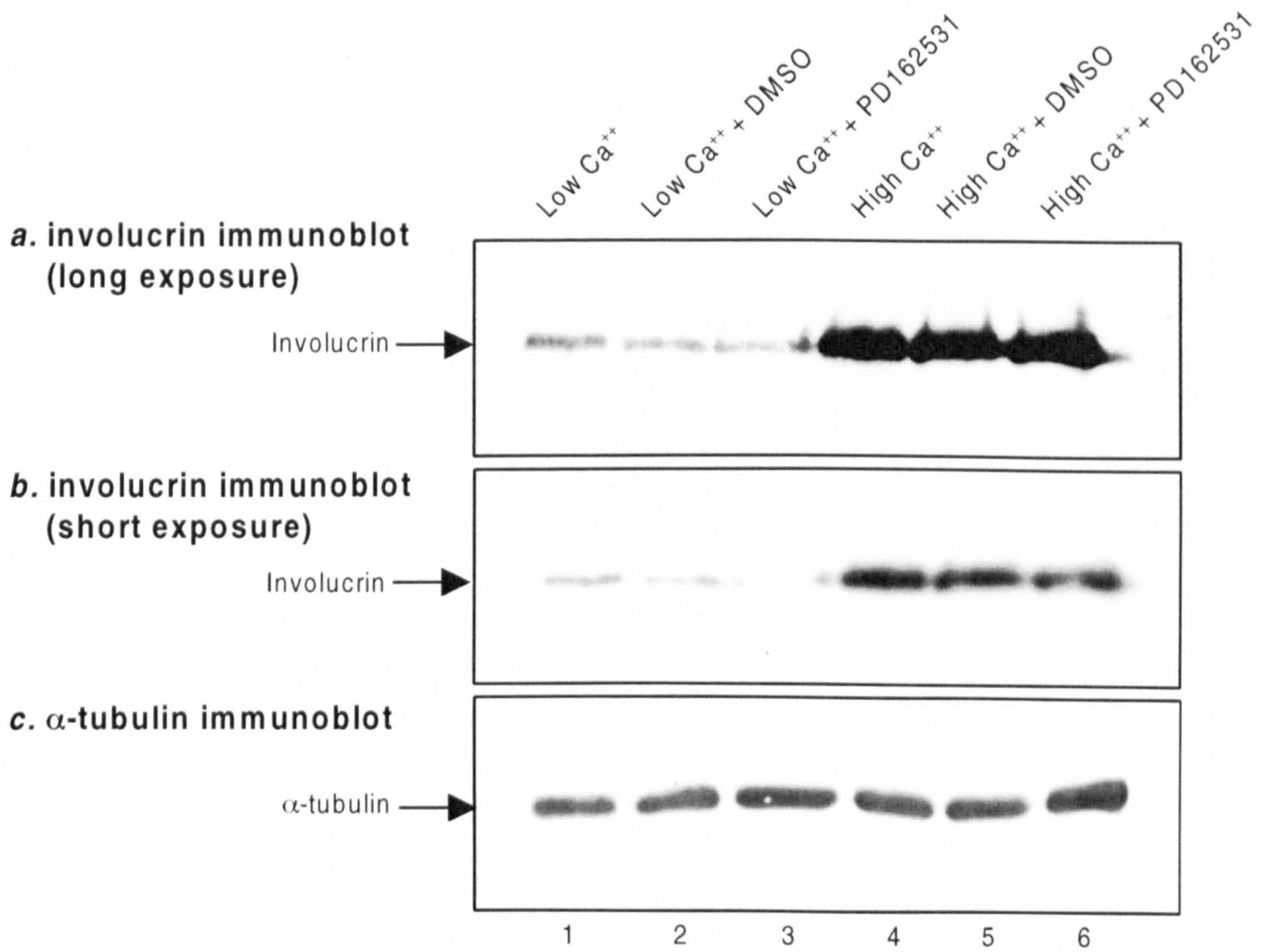


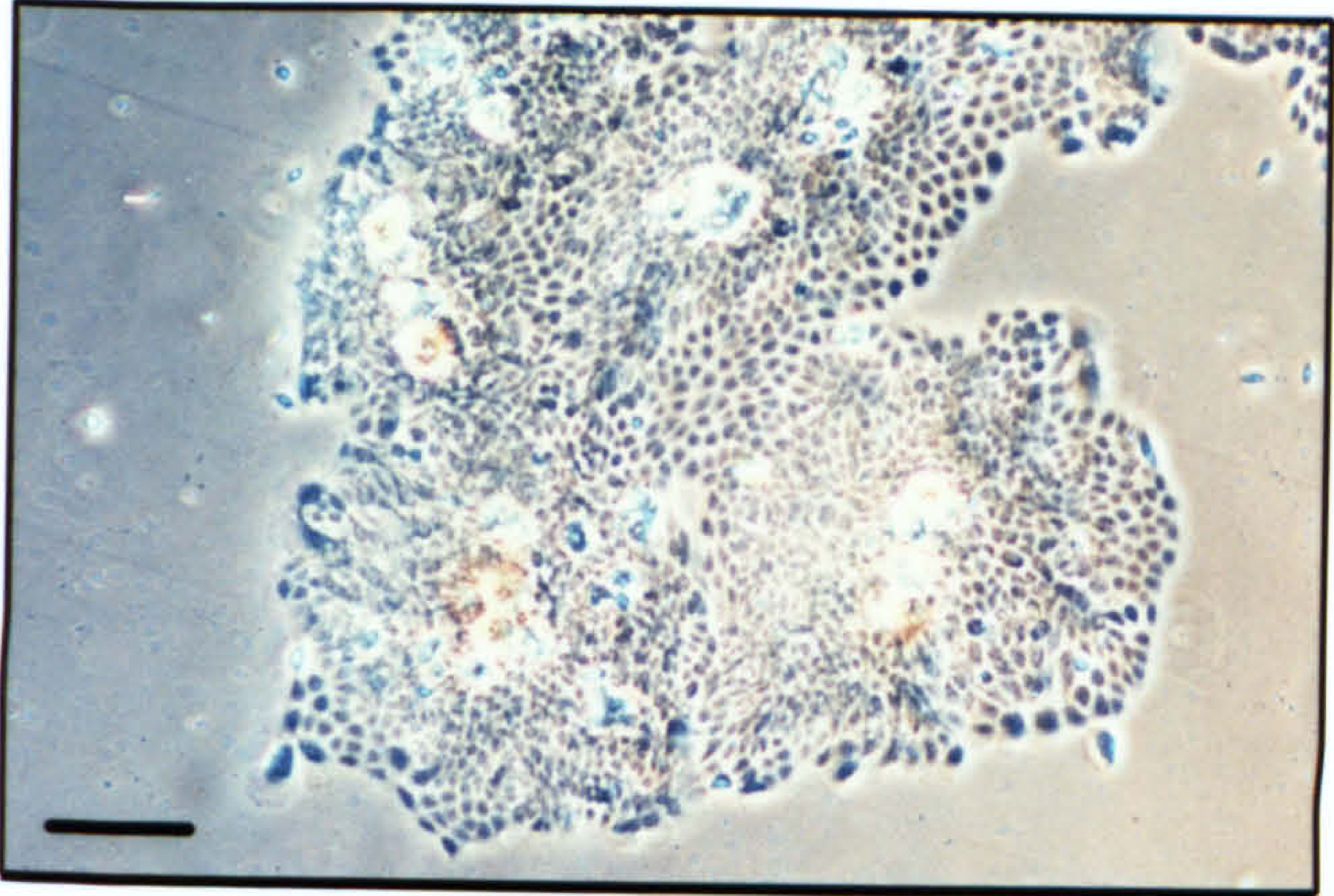
Figure 47

Figure 47
The effects of PD162531 on calcium-induced filaggrin expression

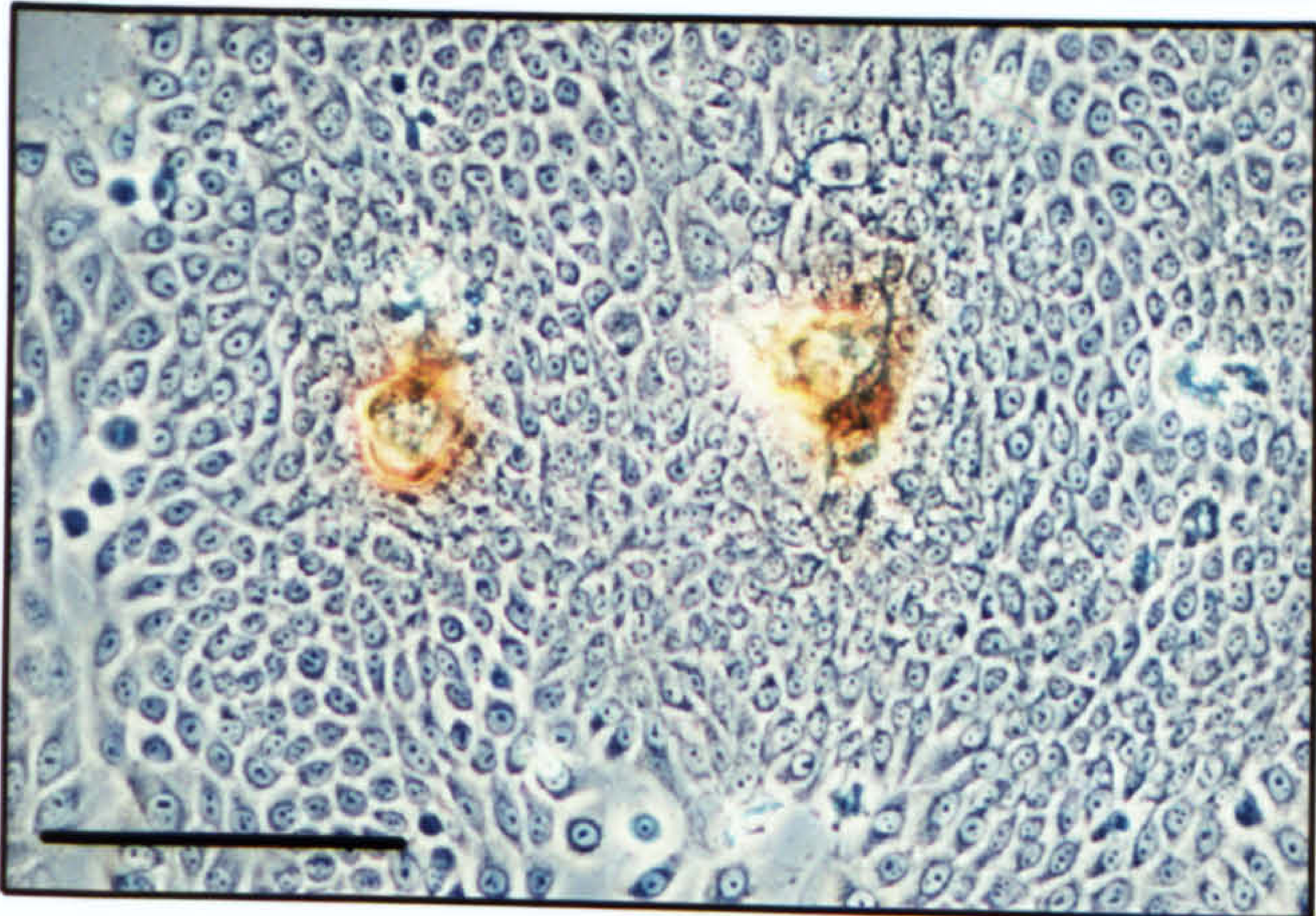
Phase-contrast photomicrographs of subconfluent adherent keratinocytes stained for filaggrin. The cells in all images were incubated in 1mM extracellular Ca^{++} for a period of 5 days in the presence of 0.02% DMSO (panels *a* and *b*) or 2μM PD162531 (panels *c* and *d*). Note that images *a* and *b* show cells maintained under the same conditions but at different magnifications, as do images *c* and *d*. The antiserum used was BT-576 diluted 1:500. Scale bars represent 200μm.

The effects of PD162531 on calcium-induced filaggrin expression

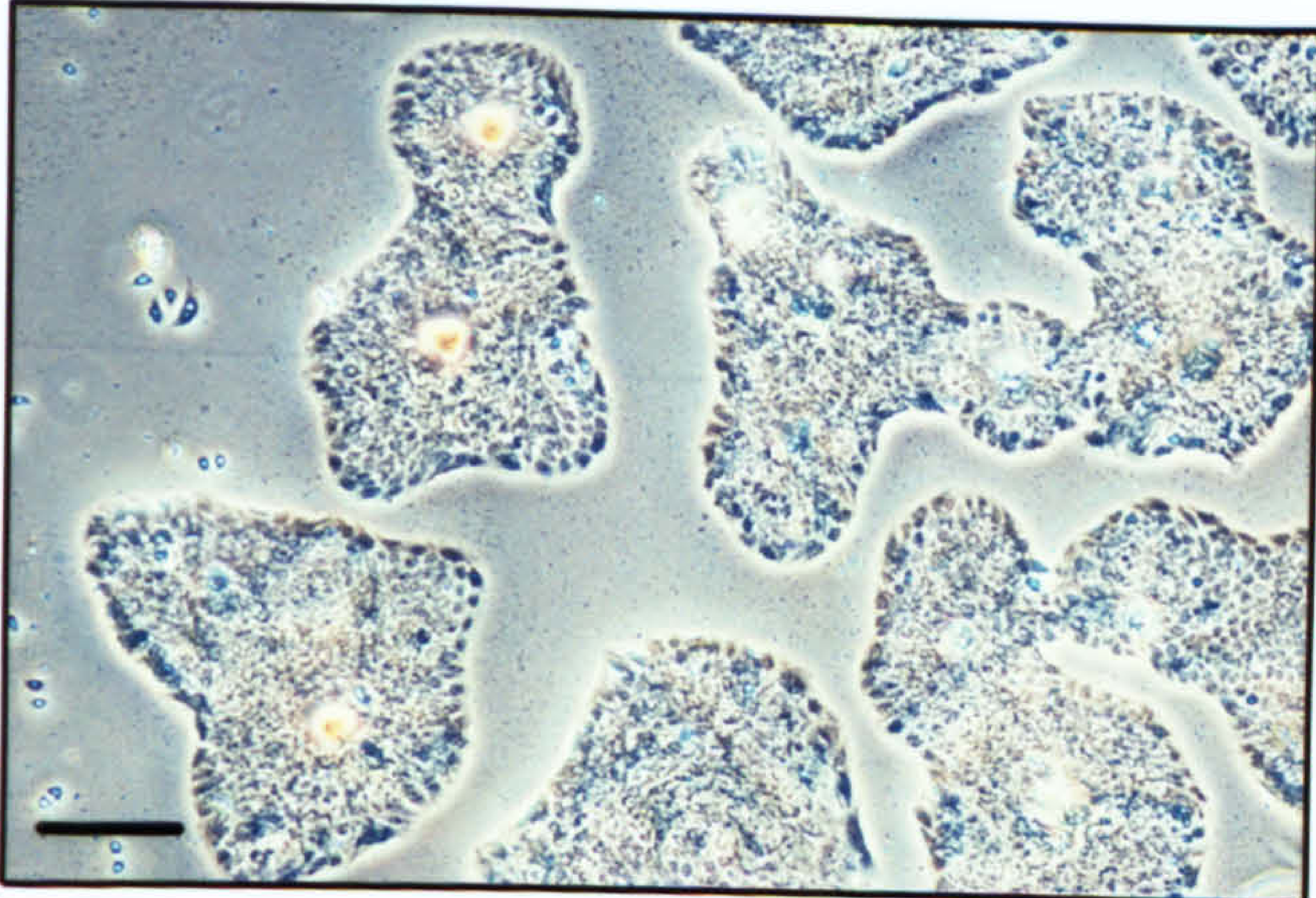
a. high extracellular calcium + DMSO



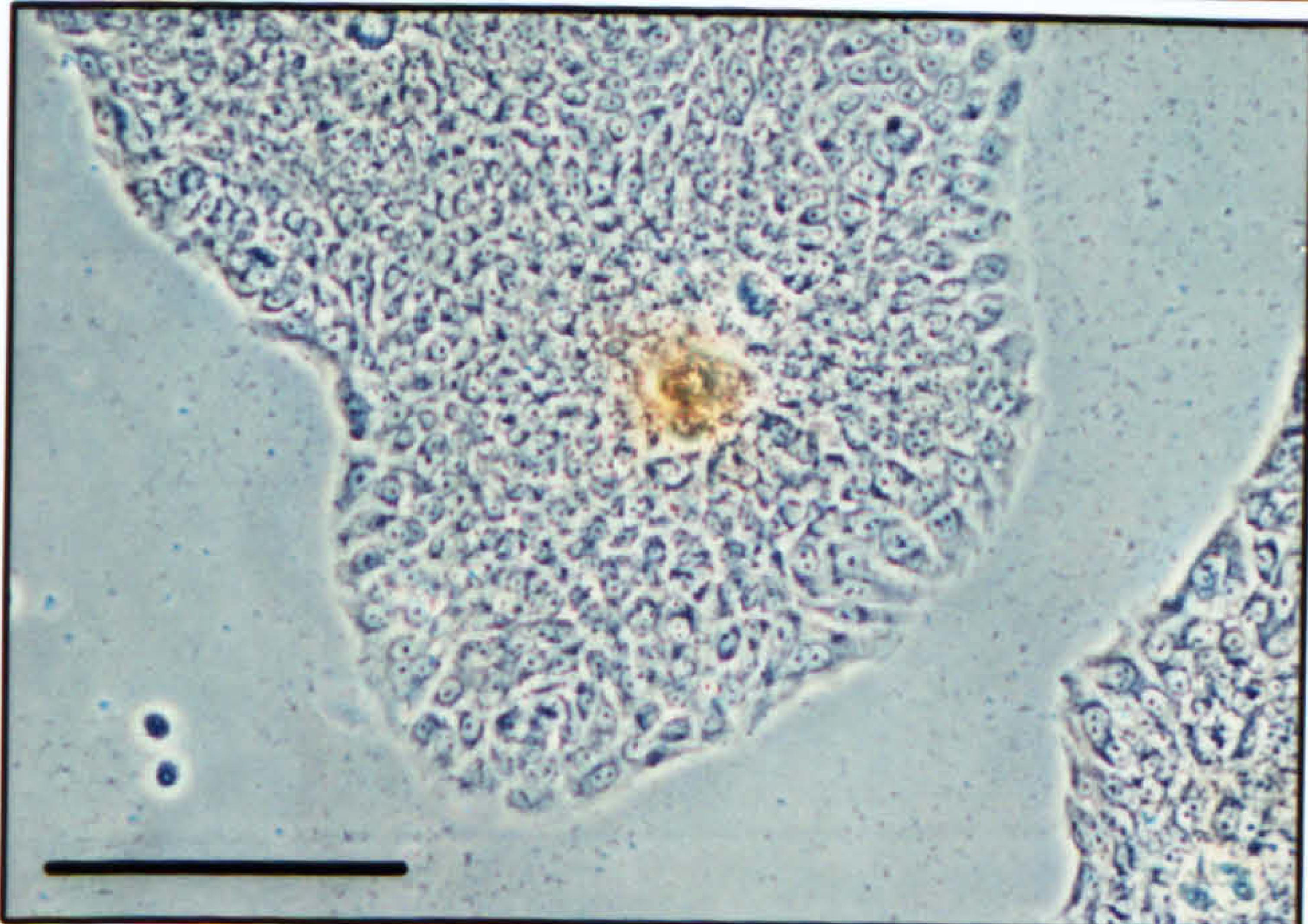
b. high extracellular calcium + DMSO



c. high extracellular calcium + PD162531



d. high extracellular calcium + PD162531



9.4 DISCUSSION

9.4.1 The role of the Src-family kinases in keratinocytes

A role for the Src kinases in the adherens junction is supported by their subcellular localisation at sites of cell-cell contact in cells switched to high extracellular Ca^{++}. However, the physical association of c-Src with either E-cadherin or $p120^{ctn}$ could not be demonstrated by co-immunoprecipitation (not shown), suggesting that any interactions between c-Src and these adhesion components are very weak or that c-Src associates with some other component of the junction.

The absence of changes in the activity of the Src-family kinases in response to extracellular Ca^{++} appears to contradict the findings of Zhao *et al.* (1992 and 1993) who demonstrated increased c-Src activity and diminished c-Yes activity during keratinocyte differentiation. However, their studies examined later stages of differentiation (cornified envelope assembly), induced by ionophore treatment, whereas the earlier phases of differentiation were examined here. Caution is required in the interpretation of *in vitro* tyrosine kinase assays as they often fail to reflect biologically significant changes in kinase activity (M. Frame, Beatson Institute).

Attempts to obtain an indirect indication of the activity of the Src-family kinases localised to adherens junctions by measuring $p120^{ctn}$ tyrosine phosphorylation suggested that these kinases may not be active during the formation of the adhesions, at least towards $p120^{ctn}$. The tyrosine phosphorylation of $p120^{ctn}$ decreased during the formation of intercellular adhesions in response to extracellular Ca^{++}, consistent with reports that increased tyrosine phosphorylation of this protein correlates with the disruption of cadherin-mediated adhesions in epithelial cells (Kinch *et al.,* 1995; Papkoff, 1997). However, the function of tyrosine phosphorylated $p120^{ctn}$ in keratinocytes remains something of an enigma. Tyrosine phosphorylated $p120^{ctn}$ was only detectable in the Triton-insoluble fraction suggesting that it may be associated with the cytoskeleton. However, the association of $p120^{ctn}$ with the actin cytoskeleton (inferred from detergent solubility) appears to vary between different cell types. For example in *ras*-transformed MCF-10A breast epithelial cells, $p120^{ctn}$ is detergent-soluble irrespective of its phosphorylation state (Kinch *et al.,* 1995). In E-cadherin-expressing L-cells, $p120^{ctn}$ phosphorylation by v-Src correlates with increased detergent solubility (Papkoff, 1997). However, neither of these cell types is likely to reflect the

situation in normal untransformed epithelial cells, such as keratinocytes, naturally expressing members of the classical cadherin family.

These experiments were performed on $p120^{ctn}$ extracted from the entire cell, representing protein localised to adhesions and also the protein from elsewhere within the cell, the latter potentially being the major fraction of cellular $p120^{ctn}$ (Papkoff, 1997). Attempts to measure the phosphotyrosine content of cadherin-associated $p120^{ctn}$ were unsuccessful (not shown). Although $p120^{ctn}$ was readily detectable in E-cadherin immunoprecipitates, tyrosine phosphorylated $p120^{ctn}$ was not. Furthermore, E-cadherin was present in $p120^{ctn}$ immunoprecipitates regardless of the presence of tyrosine phosphorylated $p120^{ctn}$ in these preparations (not shown). Taken together, these observations indicate that $p120^{ctn}$ need not be phosphorylated to bind to E-cadherin in keratinocytes. This is consistent with a report that tyrosine phosphorylated $p120^{ctn}$ cannot be detected in association with VE-cadherin in human endothelial cells regardless of the amount of tyrosine phosphorylated $p120^{ctn}$ present in the cell (Lampugnani *et al.,* 1997). However, there are also reports of phosphorylated $p120^{ctn}$ binding to E-cadherin in a carcinoma cell line (Kinch *et al.,* 1995).

9.4.2 The Src-family kinases and cell-cell adhesion

In the cells treated with the Src-family kinase inhibitor PD162531 in LKGM, the cells aggregate together and surprisingly, E-cadherin is re-localised to the sites of cell-cell contact. This indicates that treatment with the Src kinase inhibitor may induce the formation of cell-cell adhesions.

These observations suggest that the moderate levels of cadherin visualised in the cell-cell contacts are sufficient to form adhesions of adequate strength to maintain the observed morphology in the presence of PD162531. In high extracellular Ca^{++}, such an adhesion would normally be strengthened by the recruitment of more cadherin in an actin-dependent manner (section 6.5.1). Inhibiting members of the Src family could stabilise these 'proto-adhesions' which seem to exist at a much lower frequency in untreated subconfluent low extracellular Ca^{++} cultures. Therefore, inhibiting the Src-kinases may stabilise weak cadherin-mediated interactions but does not induce the strengthening of the immature adhesions by the recruitment of more cadherin (section 9.4.4). This is consistent with a role for the Src kinases in adherens junction turnover.

An alternative explanation for the altered keratinocyte distribution seen in response to treatment with PD162531 is that this compound does not affect cell-cell adhesion *per se,* but that it inhibits migration. Under these circumstances, cells would remain where they were generated by mitosis. This is unlikely since the aggregation of the cells in response to the inhibitor occurs within hours and involves the rearrangement of existing cells rather than the *de novo* formation of tightly packed aggregates of newly produced cells.

In vitro, the Src-kinases may turn over the transient cell-cell adhesions which are occasionally seen between cells maintained in low extracellular Ca^{++}. The three members of the Src-family kinases that are present in human keratinocytes (c-Src, Fyn and c-Yes) are incorporated into adherens junctions at an early stage, but may not become active until the turnover of the adhesion is required. This process could specifically involve the action of the Src kinases at the cell-cell adhesion since it has been shown that Src-induced epithelial cell scattering is independent of transcription, and is likely to involve changes in the cytoskeleton or cytoskeleton-associated molecules (Boyer *et al.,* 1997).

In normal adult human epidermis, c-Yes is not present at the cell-cell adhesions (Krueger *et al.,* 1991). However, in epidermis adjacent to a wound, c-Yes is localised to the keratinocyte cell-cell adhesions, particularly in the spinous layer. This is consistent with a role for c-Yes in the turnover of cell-cell adhesions when keratinocytes need to break free from neighbouring cells to migrate into the wound site. Also, in human endothelial cell cultures, where cell-cell adhesion is mediated by VE-cadherin, established cell-cell adhesions contain low levels of phosphotyrosine. However, if the culture monolayer is wounded, the adherens junctions of cells close to the wound site become tyrosine phosphorylated before the cells sever their contacts with their neighbours and become migratory (Lampugnani *et al.,* 1997). This may indicate that tyrosine kinases such as members of the Src-family are activated at this time. Taken together, these data indicate that the most likely function of the Src-family kinases at adherens junctions may be to control the turnover of these adhesions when cells become motile. Thus, the Src kinases may play an important role during processes such as embryogenesis and wound healing when the regulated disruption of adherens junctions is required to permit cells to break free from their neighbours.

9.4.3 The Src-family kinases and keratinocyte differentiation

An alternative function for the Src-family kinases localised to cell-cell adhesions is in the transduction of signals from the junction into the cell, a process that is not well understood. The data presented in Chapters 6 and 8 showed that differentiation can be induced by the formation of adherens junctions. Therefore we tested whether inhibiting the Src kinases could delay or inhibit the expression of differentiation-specific proteins in response to high extracellular Ca^{++}.

Treating the cells with PD162531 had no effect on the Ca^{++}-induced expression of involucrin or filaggrin. Furthermore, the expression of involucrin in cells maintained in LKGM was also unaffected by treatment with the Src kinase inhibitor. Studies using mice nullizygous for *c-src, fyn* and *c-yes* have shown that Fyn is involved in keratinocyte differentiation but c-Src and c-Yes are not essential. In the *fyn* -/- mice, the expression of involucrin in response to Ca^{++} was not affected so it is perhaps no surprise that treating the cells with PD162531 had no effect on involucrin induction. However, in both the *fyn* -/- mice and the keratinocytes derived from these animals, filaggrin expression was delayed during differentiation (Calautti *et al.*, 1995). Unfortunately, we were unable to quantify the proportion of cells expressing filaggrin after treatment with and without Src kinase inhibitor. No major differences were apparent when the stained cells were viewed using a phase-contrast microscope. These data cannot be interpreted fully until the effects of the Src kinase inhibitor on the proportion of cells expressing filaggrin are determined accurately.

A question which arises from these observations is why the cell-cell adhesions induced by treatment with PD162531 in LKGM, which appear almost identical to those formed between confluent cells, should fail to induce differentiation. The most likely explanation for this is that the adhesions formed in response to the inhibitor do not provide a sufficiently strong stimulus to initiate differentiation, perhaps because the inhibitor interferes with some aspect of signalling from the adhesion. However, in high extracellular Ca^{++} the inhibitor may be insufficient to block the stronger signal originating from the adherens junctions. This hypothesis could be tested by treating confluent keratinocytes with PD162531 and then investigating the expression of involucrin and filaggrin in these cells. If the inhibitor has a partial effect on signalling

from the adhesions, the expression of these differentiation-specific proteins may not be increased under these conditions.

9.5 SUMMARY

Taken together, the data presented in this chapter indicate that the Src-family kinases in human keratinocytes are most likely to be involved in the regulation of the disassembly of cadherin-mediated adhesions and that they may not play an important role in signalling differentiation from these adhesions. The modulation of keratinocyte adherens junction stability is represented by the schematic diagram (Figure 48). Three 'states' of adhesion are shown on the diagram, 'weak unstable', corresponding to the sporadic adhesions which are sometimes seen between subconfluent cells maintained in LKGM; 'weak stable', corresponding to the adhesions seen between cells treated with PD162531 in LKGM (and possibly between post-confluent cells maintained in LKGM); and 'strong', corresponding to the adhesions seen between cells incubated in high extracellular Ca^{++} where large amounts of cadherin are localised to the junction. The amount of E-cadherin that would be localised to the cell-cell contacts is represented on the diagrams below the model and is the same for both types of 'weak' adhesion.

In the model, the cell-cell adhesions exist in a state of dynamic equilibrium which can be shifted in favour of a particular type of adhesion by extracellular and intracellular factors. For example, inhibiting the Src-kinases changes the 'weak unstable' form of adhesion to the 'weak stable' adhesion. Switching to high extracellular Ca^{++} causes the formation of weak cell-cell adhesions but does not produce a 'strong' adhesion unless a normal actin cytoskeleton is present. The 'weak' adhesions may only involve cadherin which is constitutively present in the cell membrane in LKGM whereas the 'strong' adhesion requires the actin-dependent translocation of additional cadherin to the cell-cell junction. This preliminary model requires further testing, including the possible turnover of 'strong' adhesions by elevated Src kinase activity which will be a subject of future studies (section 10.7.2).

This model for the modulation of adherens junction stability by the Src kinases is consistent with reports of elevated Src kinase activity in some invasive human cancers, for example c-Src and c-Yes in colorectal carcinoma (Skoudy *et al.,* 1996; Pena *et al.,*

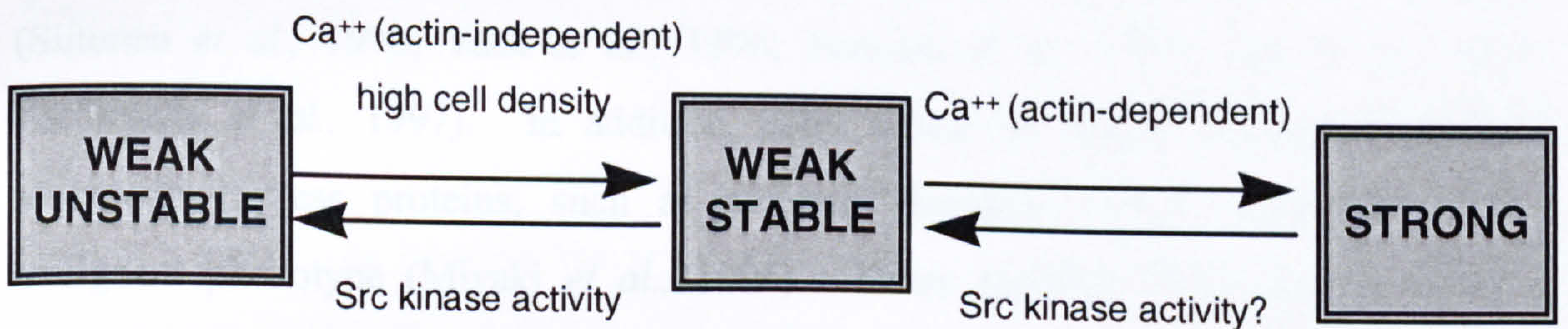
1995; Cartwright *et al.,* 1990). The importance of these changes in Src activity was demonstrated by the expression of antisense c-Src in the HT 29 colorectal carcinoma cell line, resulting in reduced tumourigenicity in nude mice (Staley *et al.,* 1997). Thus, the disruption of adherens junctions by Src-family kinase mediated tyrosine phosphorylation could occur *in vivo*, not only permitting tissue remodelling during development and wound healing, but also contributing to the aberrant properties of malignant cells.

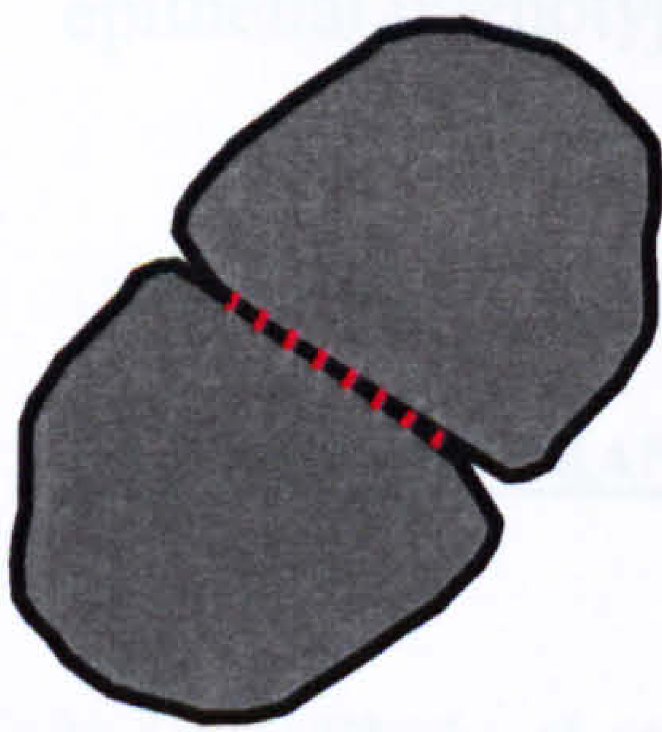
Figure 48

Figure 48
A model for the regulation of adherens junction stability

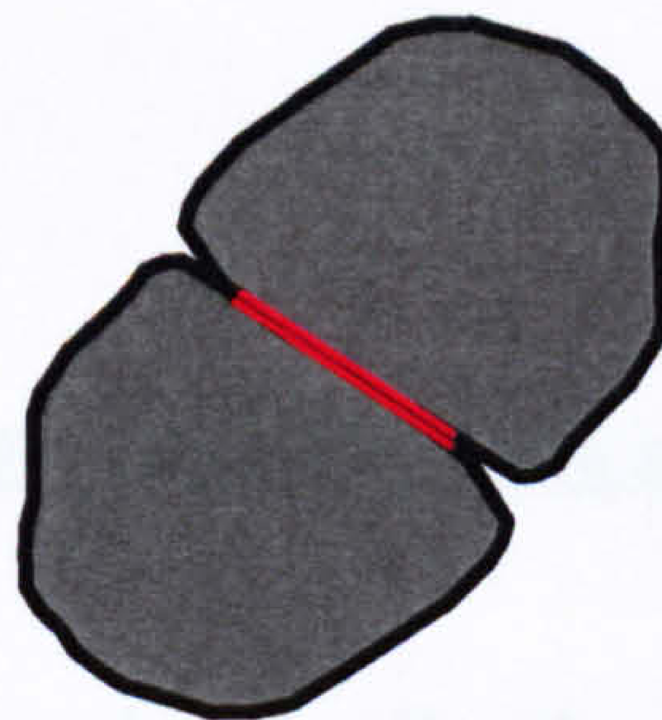
This schematic diagram represents the dynamic equilibrium of adherens junction stability that exists in human keratinocytes *in vitro*. The different levels of junction stability are 'weak unstable', 'weak stable', and 'strong'. The lower diagrams represent pairs of keratinocytes (grey), indicating the amount of E-cadherin that would be localised to the cell-cell contact areas (red) in each form of adhesion.

A model for the regulation of adherens junction stability

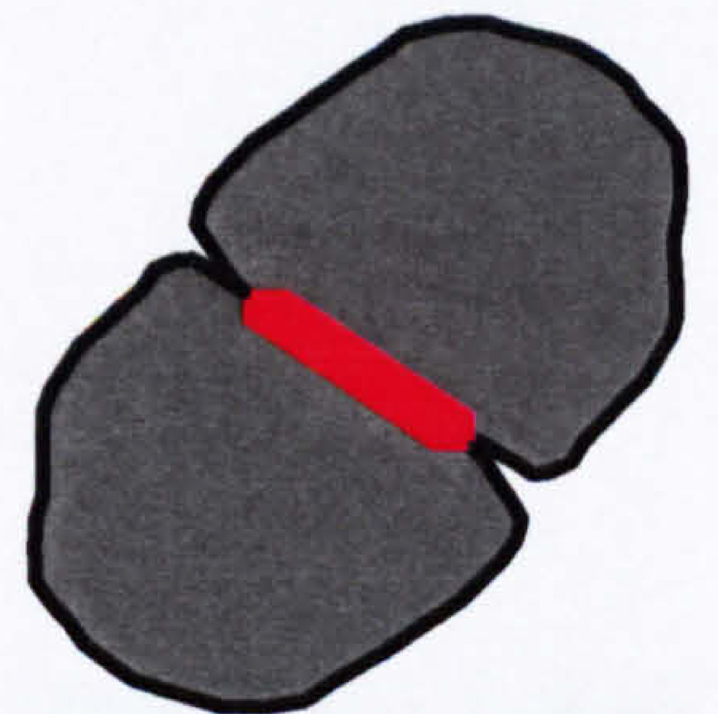




e.g. low
extracellular Ca^{++}
(very infrequent)



e.g. low
extracellular Ca^{++}
+ PD162531



e.g. high
extracellular Ca^{++}

CHAPTER 10
GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PROSPECTS

10.1 THE ORIGIN OF THE CALCIUM-INDUCED SIGNAL

The data presented in this study are supportive of a role for the keratinocyte cadherins in mediating Ca^{++}-induced differentiation *in vitro*. This conclusion is consistent with reports of impaired differentiation in a variety human carcinomas, including squamous cell carcinoma, often coinciding with reduced or undetectable expression of E-cadherin (Siitonen *et al.*, 1996; Tada *et al.*, 1996; Tomson *et al.*, 1996; Hao *et al.*, 1997; Zschiesche *et al.*, 1997). In addition, cells which no longer express E-cadherin aberrantly express proteins, such as secreted proteases, which contribute to the malignant phenotype (Miyaki *et al.*, 1995). Taken together, these studies strongly implicate the cadherins, and in particular E-cadherin as critical mediators of the epithelial phenotype.

10.2 SIGNAL TRANSDUCTION FROM THE ADHERENS JUNCTION

It is unlikely that cadherin-mediated cell-cell adhesion *per se* is sufficient to induce keratinocyte differentiation. However, signals originating from these cell-cell adhesions are likely to trigger the differentiation program. Signalling from the adherens junction is not well understood but is likely to be important given the profound influence adherens junctions have on cellular behaviour. Potential mediators of downstream signalling from the keratinocyte adherens junction include the adaptor protein Shc (section 2.1.1B), recently shown to bind to N-cadherin (Xu *et al.*, 1997a); β-catenin, a protein which has roles both in cell-cell adhesion and in signalling; and members of the Src-family kinases which are localised to keratinocyte adherens junctions and are involved in signalling from the focal adhesion (Schlaepfer and Hunter, 1996). The potential roles of these different signalling mediators will now be considered in more detail.

10.2.1 Signal transduction *via* β-catenin

A. β-catenin as a signal transducer in epithelial cells

β-catenin is a molecule with multiple functions and can be present in a number of locations within the cell, including the cell-cell adhesions, the cytoplasm and the nucleus (Huber *et al.,* 1996). In addition to its function in mediating interactions between α-catenin and the cadherins, β-catenin is also involved in transducing signals initiated by the Wnt growth factors. It forms complexes with the Lef-1 (lymphoid enhancer factor-1) transcription factor and with members of the Tcf-(T-cell factor) family of transcription factors thereby altering their activity (McCrea *et al.,* 1993; Behrens *et al.,* 1996; Molenaar *et al.,* 1996; Korinek *et al.,* 1997). These transcription factors are members of the HMG (high mobility group) family which cause DNA bending, thought to permit access to the DNA by other transcription factors including Ets-1, PEPB2α and ATF/CREB (Giese *et al.,* 1992; Love *et al.,* 1995). In *Drosophila,* there is a correlation between the levels of cytoplasmic Armadillo (the *Drosophila* β-catenin homologue) and cell fate, illustrating the importance of β-catenin-mediated signal transduction (Riggleman *et al.,* 1990; Peifer *et al.,* 1994). Furthermore, injecting β-catenin mRNA into *Xenopus* embryos induces the formation of a second body axis (Funayama *et al.,* 1995).

Excessive activity of the β-catenin/Lef-1 and β-catenin/Tcf-4 complexes has been demonstrated in colon carcinoma cell lines which often have high levels of uncomplexed β-catenin. These catenin/transcription factor complexes are capable of binding to the E-cadherin promoter thereby reducing E-cadherin expression and perhaps contributing further to the aberrant behaviour of these cells (Huber *et al.,* 1996). The elevated β-catenin levels in malignant cells are frequently due to dysfunctional adenomatous polyposis coli (APC) protein which normally regulates cytoplasmic β-catenin levels, or to alterations within β-catenin which increase its stability (Groden *et al.,* 1991; Rubinfeld *et al.,* 1993; Munemitsu *et al.,* 1995; Korinek *et al.,* 1997; Rubinfeld *et al.,* 1997). APC binds to the *armadillo* repeats of β-catenin (Hülsken *et al.,* 1994) and is present in the cytoplasm and nuclei of epithelial cells (Neufeld and White, 1997). Normally, β-catenin phosphorylated in the amino-terminal region by glycogen synthase kinase-3β (GSK3β, a serine/threonine kinase) binds to APC and is

subsequently targeted for degradation (Munemitsu *et al.,* 1995; Barth *et al.,* 1997). Although the amino-terminal region of β-catenin is not involved in mediating binding with APC, it is thought to regulate the dynamics of this association in some way (Morin *et al.,* 1997). The importance of the GSK3β phosphorylation sites in β-catenin was illustrated by expressing β-catenin lacking them in MDCK cells, causing the cells to adopt a more scattered, fibroblastic phenotype (Barth *et al.,* 1997).

Defects in APC also occur in squamous cell carcinoma, and have been implicated in the development of the disease, suggesting that deregulated β-catenin could also contribute to this malignancy (Uzawa *et al.,* 1994). However, it is not clear whether the levels of uncomplexed β-catenin are elevated in these cells, nor has the expression of genes which could be modulated by a β-catenin/transcription factor complex been investigated. In squamous cell carcinoma, the expression of β-catenin is sometimes reduced, usually paralleling reduced E-cadherin expression, indicating that a general loss of adherens junction components may take place (Takayama *et al.,* 1996; Nakanishi *et al.,* 1997; Andrews *et al.,* 1997). Elucidation of the potential role of β-catenin in keratinocyte malignant transformation will require the measurement of uncomplexed β-catenin levels in normal and transformed cells.

B. β-catenin in the epidermis

The cadherin-mediated adherens junctions compete with APC for free β-catenin thereby reducing the amount available for signalling and altering the intracellular distribution of β-catenin (Hülsken *et al.,* 1994; Fagotto *et al.,* 1996; Hermiston *et al.,* 1996). Therefore, it is possible that the formation of cadherin-mediated adherens junctions in response to high extracellular Ca^{++} concentrations decreases the levels of free β-catenin in keratinocytes, thereby altering transcription and initiating the differentiation program.

Consistent with this hypothesis, β-catenin is present in all the living layers of human epidermis, including the basal layer where commitment to differentiation takes place (Haftek *et al.,* 1996; Moles and Watt, 1997). Furthermore, the Lef-1 transcription factor is expressed in cultured human keratinocytes and in the basal keratinocytes of embryonic murine epidermis (Zhou *et al.,* 1995b; Kratochwil *et al.,* 1996). However, if signalling through β-catenin is a key regulator of keratinocyte differentiation in the

epidermis, it must involve transcription factors other than Lef-1 since Lef-1 is probably not expressed in the postnatal murine epidermis (Zhou *et al.,* 1995b).

Studies to determine the role played by Lef-1 in murine development have shown that Lef-1 is most likely to be involved in skin appendage formation (Zhou *et al.,* 1995b). Targeted disruption of the *lef-1* gene results in mice with a histologically normal epidermis in which stratification and keratinisation occur normally, indicating that Lef-1-mediated transcription is not vital for normal epidermal differentiation (van Genderen *et al.,* 1994). Interestingly, body hair and whiskers are absent from the nullizygous *lef-1* mice indicating that Lef-1 plays an indispensable role in hair formation. Another transcription factor which can interact with β-catenin is Tcf-1 (T-cell factor-1). However, mice made nullizygous for the *tcf-1* gene have no defects other than a reduced number of thymocytes, indicating the specialised function of this particular transcription factor (Castrop *et al.,* 1995; Verbeek *et al.,* 1995). Thus, if signalling *via* β-catenin occurs in adult human epidermis, it must involve mechanisms other than altering the activity of Lef-1 or Tcf-1.

To test the hypothesis that it is through altering the levels of uncomplexed β-catenin that the formation of adherens junctions induces keratinocyte differentiation, it would be necessary to measure the levels of free β-catenin in keratinocytes maintained in LKGM and in cells switched to HKGM to induce the formation of adherens junctions. This could be achieved by the use of a molecule comprising the cytoplasmic domain of E-cadherin conjugated to glutathione-S-transferase which binds to uncomplexed β-catenin but not to β-catenin complexed with E-cadherin or APC (Papkoff *et al.,* 1996; Papkoff, 1997). If the hypothesis is correct, reducing the levels of uncomplexed β-catenin should induce differentiation. This provides one possible explanation for the observations of Zhu and Watt (1996) who found that expressing the cytoplasmic domain of E-cadherin in keratinocytes increased the proportion of differentiated cells. Whilst this manipulation increased the total levels of β-catenin, the authors did not measure the levels of uncomplexed β-catenin which may have been lower in the cells expressing the dominant-negative E-cadherin protein.

10.2.2 Signal transduction *via* Shc

A potential mechanism for the transduction of signals from the adherens junction into the cell involves Shc, an adaptor protein which binds to N-cadherin *via* its SH2 domain and can recruit Grb2 thereby indirectly activating Ras (Xu *et al.,* 1997a and section 2.1.1B). This association between Shc and the cytoplasmic domain of N-cadherin requires the phosphorylation of N-cadherin by c-Src, providing one explanation for the localisation of members of the Src-family kinases to adherens junctions (Tsukita *et al.,* 1991; section 9.2.1). Also, in A431 cells, the association between Shc and N-cadherin is decreased by reducing the extracellular Ca^{++} concentration, suggesting that Shc-mediated signalling could be influenced by the extracellular Ca^{++} concentration (Xu *et al.,* 1997a).

Keratinocytes mainly express the 52 and 46kD forms of Shc (Mainiero *et al.,* 1997) which have established roles in mitogenic signalling pathways involving $p21^{Ras}$ (e.g. Basu *et al.,* 1994; section 2.1.1B). However, it is also possible that Shc could mediate signals which initiate the differentiation program in keratinocytes, perhaps by causing sustained activation of $p21^{Ras}$ and hence MAP kinase.. In PC12 cells, the outcome of MAP kinase activation is determined by the duration of the MAPK response. Transient activity is mitogenic whereas prolonged activation stimulates differentiation (Marshall, 1996). We have not examined the role of Shc in Ca^{++}-induced human keratinocyte differentiation *in vitro.*

10.2.3 Signal transduction *via* the Src-family kinases

In this study, we demonstrated the presence of members of the Src-family kinases at keratinocyte adherens junctions. Members of this family of protein tyrosine kinases have previously been shown to function in signal transduction from the cell surface, e.g. Lck from the CD4 receptor, Src from focal adhesions and from growth factor receptors (Schlaepfer *et al.,* 1994; Schlaepfer *et al.,* 1996; Twamley-Stein *et al.,* 1993; Veillette *et al.,* 1988). Furthermore, studies in murine keratinocytes have indicated that Fyn is involved in the keratinocyte differentiation process and that v-Src expression inhibits differentiation (Calautti *et al.,* 1995; Weissman and Aaronson, 1985). However, the Src-family kinase inhibitor failed to affect the expression of involucrin or filaggrin in response to calcium. This does not rule out the Src kinases as potential mediators of signalling from the adherens junctions, although there is currently no evidence that the

Src kinases relay signals from these adhesions. Thus, our data are most consistent with a role for the Src kinases in the control of junction turnover, perhaps during cell motility (section 10.3).

10.3 Adherens junction stability and the Src-family kinases

The data presented in this study agree with data presented by other groups and indicate that the Src-family kinases are most likely involved in the control of cell-cell adhesion turnover (section 9.4.4). In normal epidermis, the turnover of adherens junctions may be required to permit differentiating cells to leave the basal layer and perhaps also to allow cell migration during wound healing. Although there are at least three Src-substrates present in keratinocyte adherens junctions (E-cadherin, β-catenin and $p120^{ctn}$ - Behrens *et al.,* 1993), it is not clear how their phosphorylation affects junction stability. Studies have indicated that the phosphorylation of β-catenin and E-cadherin does not cause them to dissociate from one another although it is possible that their interactions with the actin cytoskeleton may be perturbed (Takeda *et al.,* 1995; Papkoff, 1997).

10.4 Protein kinase C and keratinocyte behaviour

Members of the protein kinase C family of enzymes are implicated in keratinocyte differentiation, particularly the PKCβ and PKCη isozymes. PKCβ is normally expressed in the upper layers of the epidermis but is virtually absent from psoriatic epidermis, suggesting that it may be involved in the control of keratinocyte proliferation or differentiation (Osada *et al.,* 1993). Furthermore, the PKCη isozyme may be involved in the granular to squamous transition in murine epidermis (Kuroki *et al.,* 1997). Artificially activating PKC using PMA affects human keratinocyte differentiation *in vitro,* promoting features of terminal differentiation including cornified envelope assembly (Parkinson and Emmerson, 1982).

PKC is also implicated in the regulation of keratinocyte cell-cell adhesion. Activating PKC using PMA, *in vitro,* induces the formation of adherens junctions.

Furthermore, PKC function is required for the formation of adherens junctions in response to high extracellular Ca^{++} concentrations (Lewis *et al.,* 1994a). Thus, in keratinocytes, PKC may be involved both in the formation of adherens junctions, perhaps having an opposite role to the Src kinases, and in the regulation of differentiation. More detailed analyses are required to separate the functions of the various PKC isozymes present in keratinocytes.

10.5 CELL ADHESION AND *IN VIVO* DIFFERENTIATION

A number of studies have shown that depriving keratinocytes of integrin-mediated matrix contact can initiate differentiation (Green, 1977; Watt *et al.,* 1988). In the *in vitro* system used for this study, cell-cell adhesion can also modulate keratinocyte differentiation. Thus, both the formation of cadherin-mediated cell-cell adhesions and the loss of integrin-mediated matrix contacts can induce keratinocyte differentiation. The possibility of a relationship between these two phenomena now arises and is discussed below.

In the basal layer of the epidermis, some cells commit to terminal differentiation whilst others continue to proliferate, a decision which could be made on the basis of signals each cell receives from its adhesions. One possibility is that cell-matrix adhesions provide a signal which inhibits differentiation and in the case of the hemidesmosome is also mitogenic (Mainiero *et al.,* 1997). Conversely, the cell-cell adhesions produce a signal which promotes differentiation. In this model, shown schematically in Figure 49, the decision to differentiate would be made on the basis of the relative strengths of the signals originating from the cell-matrix and cell-cell adhesions. Thus, in cells totally deprived of cell-matrix contacts under artificial conditions, the 'default' response is differentiation. In adherent cells induced to form cell-cell adhesions, some cells receive a sufficiently strong signal from the adherens junctions that the signals originating in the cell-matrix adhesions are overridden and the cell differentiates. Supportive of this hypothesis, variations in the levels of E-cadherin and integrins in the basal layer of the epidermis have been reported (Moles and Watt, 1997). Interestingly, an inverse relationship between cadherin levels and integrin levels exists, indicating that some cells may be more likely to differentiate (high cadherin, low

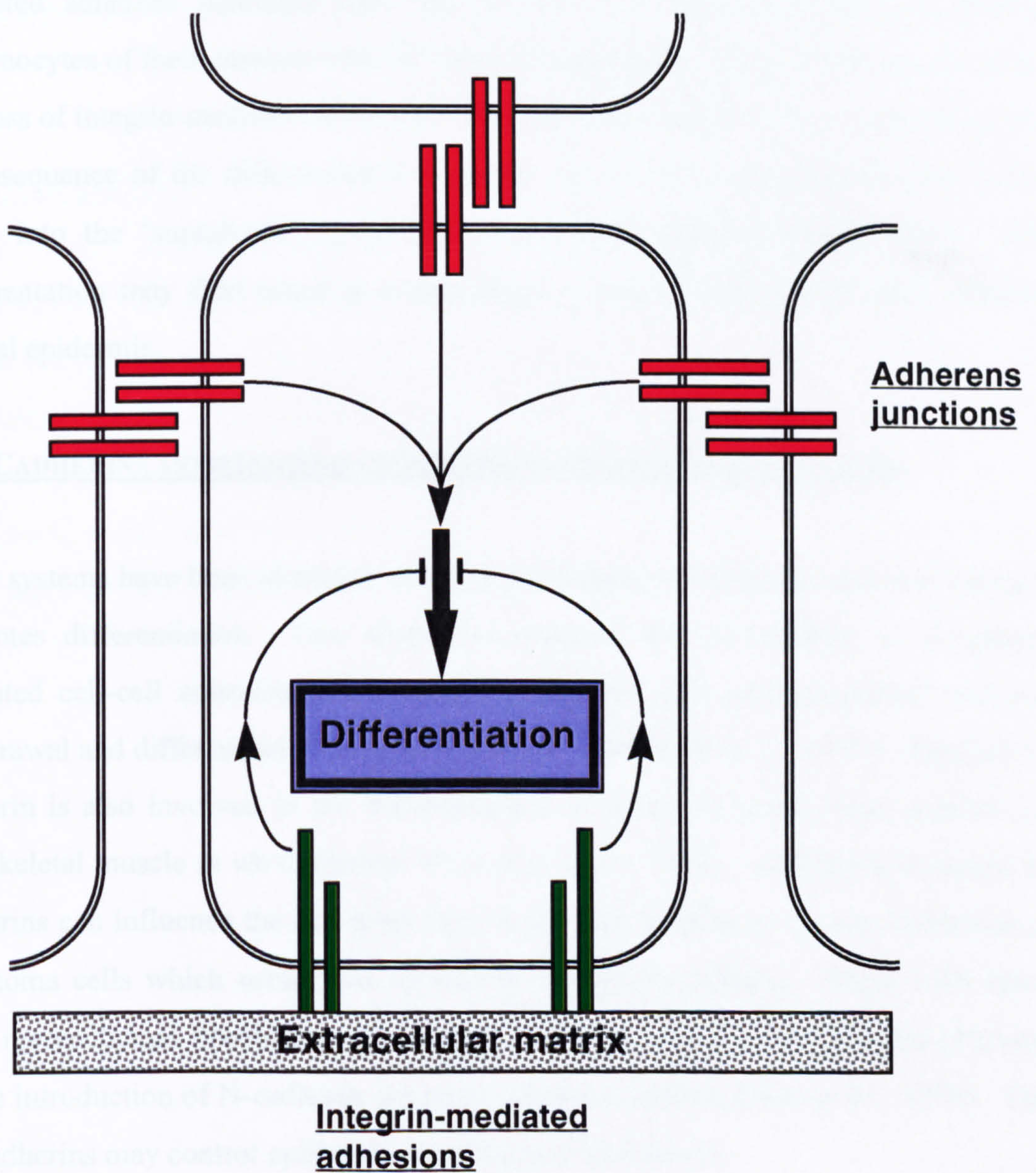
integrin) whilst others are more likely to proliferate (low cadherin, high integrin), if this hypothesis is correct. There are reports that the stem cells in the epidermis express high levels of integrins and it is possible that the levels of integrin expressed by the daughter cells may progressively decline as they progress through the transit-amplifying stages (Jones *et al.*, 1995). Conversely, cadherin expression may simultaneously increase in these cells, which would then reach a state of differentiation-competence after completing a certain number of divisions. Once differentiation has been initiated, the cells then lose integrin function, facilitating their expulsion from the basal layer of the epidermis (Hodivala and Watt, 1994).

The mechanism by which detachment from the extracellular matrix induces aspects of differentiation is not clear. One explanation is that depriving keratinocytes of their contacts with the matrix causes detachment-induced cell death, or anoikis (Frisch and Francis, 1994), and that a consequence of this is that the cells express some differentiation-specific proteins such as involucrin. Under these circumstances, the expression of these differentiation-specific proteins would be a component of a specialised apoptotic process, perhaps evolved to prevent the proliferation of keratinocytes prematurely detached from the basement membrane. Supportive of this hypothesis, suspending keratinocytes in methyl-cellulose induces features of apoptosis including DNA degradation and a reduction in the abundance of Bcl-x_L (Rodeck *et al.*, 1997). However, it is also possible that normal *in vivo* differentiation has certain features in common with apoptosis. Keratinocytes in the spinous and granular layers of adult epidermis contain fragmented DNA, indicating that they may be undergoing apoptosis during these phases of the differentiation process (Polakowska *et al.*, 1994). However, DNA fragmentation occurs at a much later time than involucrin expression is first seen *in vivo*. In contrast, in cells induced to differentiate by suspension in methyl-cellulose, DNA degradation is apparent after 24 hours, at the time when increased involucrin expression is first seen (Rodeck *et al.*, 1997; Kubler *et al.*, 1991; Nicholson and Watt, 1991). Furthermore, when cells are deprived of their matrix contacts, Bcl-x_L protein levels begin to fall after only 4 to 6 hours, at the time when involucrin mRNA levels have increased only very slightly (Nicholson and Watt, 1991).

Figure 49

Figure 49
Possible adhesive influences on keratinocyte differentiation

A model for the influences of cell-cell and cell-matrix adhesion on keratinocyte differentiation in the basal layer of the epidermis. Signals originating from adherens junctions promote differentiation whereas signals originating from integrin-mediated adhesions inhibit differentiation. The outcome depends on the relative strengths of these opposing signals.


Possible adhesive influences on keratinocyte differentiation
Adherens junctions
Differentiation
Extracellular matrix
Integrin-mediated adhesions

Taken together, these data indicate that artificially depriving keratinocytes of matrix contacts may induce the expression of differentiation-specific proteins by causing a specialised form of apoptosis since features of apoptosis and early differentiation appear almost simultaneously. Although DNA fragmentation may also be a normal component of differentiation, this does not occur until relatively late in the differentiation process. This suggests that differentiation induced by suspension culture is not equivalent to *in vivo* differentiation.

No evidence that treating keratinocytes with high extracellular Ca^{++} concentrations induced apoptosis was obtained in this study, indicating that the formation of cadherin-mediated adherens junctions does not provide a stimulus equivalent to depriving keratinocytes of their contacts with the extracellular matrix. Thus, in this model system, any loss of integrin-mediated adhesions with the extracellular matrix is most likely to be a consequence of the differentiation program, thereby allowing differentiated cells to move into the 'suprabasal' layers of the groups of adherent keratinocytes. DNA fragmentation may then occur at a later stage in these differentiated cells, similar to normal epidermis.

10.6 CADHERINS AS MEDIATORS OF DIFFERENTIATION IN OTHER SYSTEMS

Other systems have been identified where the formation of cadherin mediated adhesions promotes differentiation. One study demonstrated that N-cadherin or E-cadherin-mediated cell-cell adhesions between BHK fibroblast-like cells stimulated cell-cycle withdrawal and differentiation into skeletal muscle (Redfield *et al.*, 1997). Similarly, N-cadherin is also involved in the differentiation of primitive streak stage epiblast cells into skeletal muscle *in vitro* (George-Weinstein *et al.*, 1997). Additional evidence that cadherins can influence the cell phenotype came from studies on human squamous cell carcinoma cells which sometimes aberrantly express N-cadherin. These cells have a more mesenchymal fibroblastic phenotype which is reverted to an epithelial phenotype by the introduction of N-cadherin antisense oligonucleotides (Islam *et al.*, 1996). Thus, the cadherins may control epithelial-mesenchymal transitions.

It has also been demonstrated that expressing different cadherins in embryonic stem (ES) cells can cause the differentiation of these cells into different tissue types (Larue *et al.*, 1996). This cell type normally expresses E-cadherin initially, and when

injected into syngeneic mice, produces a teratoma consisting of a variety of different tissue types, e.g. bone, epithelium. However if these cells are made nullizygous for the E-cadherin gene, they are unable to form any organised structures within the teratoma. In addition, these cells express the mesenchymal gene, *T-brachyury* (Herrmann, 1991). Conversely, if these cells are made to express E-cadherin constitutively, they differentiate into exclusively epithelial structures in the teratoma and no longer express *T-brachyury*. A similar experiment using N-cadherin instead of E-cadherin produces cells which only differentiate into cartilage and neuroepithelium and express *T-brachyury*. Thus, an emerging theme is that the cadherins expressed by a cell exert a strong influence on its phenotype.

10.7 Conclusions

In this study I sought to define the role of the Src kinases in Ca^{++}-induced human keratinocyte differentiation *in vitro*. The data presented are more consistent with a role for the Src kinases in regulating adherens junction turnover but do not exclude a role in modulating differentiation *in vivo*. In addition, I have identified the cadherin-mediated adherens junctions as the likely origin of the signal which initiates the differentiation program in response to high extracellular Ca^{++} concentrations *in vitro*, consistent with other reports of the cadherins controlling differentiation.

10.8 FUTURE PROSPECTS

The data generated in this study have indicated a number of aspects of cell adhesion and keratinocyte behaviour which invite further investigation. As a result of these findings, we have obtained a one year strategic project grant from the Medical Research Council to continue this work. Thus, I will extend our cadherin experiments and further define the role of the Src kinases at adherens junctions as detailed below.

10.8.1 The role of the cadherins in keratinocyte differentiation

A. Cadherin clustering

To extend the cadherin clustering experiments, more quantitative analyses of the induction of differentiation-specific proteins by cadherin clustering will be carried out. In the first instance, immunoblots or ELISAs for involucrin and other differentiation-specific proteins will be performed. A more suitable approach to clustering the cell-surface cadherins, making efficient use of the antisera available, would be to coat polystyrene beads with DECMA-1 (or HECD-1) or anti-P-cadherin (NCC-CAD-299) as described in Braga *et al.,* (1997) and then using these beads to directly cluster cell-surface cadherins. In the aforementioned study, very clear E-cadherin clustering on the cell surface was induced by such beads. Additional analyses of cells treated to cluster E-cadherin and P-cadherin will also be performed, including timecourse studies to follow the kinetics of the induction of a range of differentiation-specific proteins, cell viability assays by dye exclusion or clonogenic assays and the quantification of any apoptosis using TUNEL labelling (Gavrieli *et al.,* 1993).

B. Cadherin blocking

The cadherin blocking experiments will also be extended in the near future. The effects of the blocking peptide, derived from avian N-cadherin, suggest that it may interfere with the function of both keratinocyte E-cadherin and P-cadherin. However, recent reports from other investigators have shown that peptides identical to cadherin recognition sequences are much more effective at blocking the cadherin to which they are homologous than are other closely related cadherins (Willems *et al.,* 1995). Thus, a more effective suppression of cell-cell adhesion in keratinocytes may be achieved using a cocktail of specific E-cadherin and P-cadherin blocking peptides. Furthermore,

treating the cells with only one of these specific peptides might allow the roles of E- and P-cadherin in this process to be distinguished. These peptides are currently being synthesised.

The potential toxic effects of the cadherin-blocking peptides will also be investigated. A simple and effective assay of cell viability would be to determine the reversibility of the effects of the peptides. This will be achieved by treating cells with the blocking peptides in HKGM and then rinsing the cells to remove the peptide after a suitable incubation time. At this stage, viable cells will aggregate, differentiate and stratify in response to high extracellular Ca^{++}.

The proportion of differentiated cells in peptide-treated cultures was not determined due to the difficulty of counting stratified keratinocytes (section 8.3.2). Immunoblotting for differentiation-specific proteins would provide a more quantitative measure of the effect of the peptides on keratinocyte differentiation but would require excessive amounts of peptide to treat the cells. Therefore, the amounts of differentiation-specific proteins expressed in the presence and absence of cadherin-blocking peptide will be quantitated by ELISA which will require the treatment of fewer cells than immunoblotting.

10.8.2 The role of the Src kinases in keratinocytes

The work on the role of the Src kinases in keratinocyte adhesion and differentiation will be extended, using different approaches to fully characterise the role played by these kinases. To investigate the regulation of adherens junction stability by c-Src, Fyn and c-Yes individually, keratinocytes will be prepared from neonatal *c-src*, *fyn* and *c-yes* nullizygous mice (Soriano *et al.,* 1991; Stein *et al.,* 1992; Stein *et al.,* 1994). These cells will be used in aggregation assays in the presence of low and high extracellular Ca^{++} to determine the stability of the cell-cell adhesions, and will be compared with cells derived from normal littermates. The specificity of the cell-cell adhesions will be ascertained by the use of blocking antisera or peptides.

The severing of adherens junctions will also be investigated by inducing nullizygous cells to aggregate with a relatively brief incubation in high extracellular Ca^{++} and then returning these cells to low extracellular Ca^{++}. In the absence of the function of a kinase required to turn over the adhesion, it is likely that the adhesions will

remain intact even in low extracellular Ca^{++}. This will allow the involvement of the Src kinases in keratinocyte adherens junction turnover to be verified.

To determine the structural domains of Src required for Src-induced disassembly of cell-cell adhesions we will use chicken embryo fibroblasts (CEF) which utilise N-cadherin to form intercellular adhesions at high density (Hatta and Takeichi, 1986). In the presence of constitutively active v-Src, these N-cadherin-mediated adhesions become unstable (Hamaguchi *et al.,* 1993). v-Src with mutated myristylation sequence, kinase, SH2 and SH3 domains have been generated in our laboratory from existing temperature-dependent v-Src variants. The use of these mutants may also allow the identification of the adhesion components with which Src functionally interacts during the process of adherens junction turnover.

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