2.3. Calcium signaling

Ca^{2+} is an universal signal transduction element in cells modulating cell growth and differentiation (Lodish *et al.* 2000). The calcium levels outside cells are 10 000 times higher than free intracellular Ca^{2+}. However, free $[Ca^{2+}]_i$ is the physiologically active form of calcium (Rasmussen 1988). The level of free intracellular calcium ($[Ca^{2+}]_i$) is regulated and maintained as low (~100 nM) through the action of a number of binding proteins and ion exchange mechanisms. Each cell has a unique set of Ca^{2+} signals to control its function. Ca^{2+} signal transduction is based on rises in free cytosolic Ca^{2+} concentration. Ca^{2+} can flow from the extracellular space or be released from intracellular stores. The endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Recent studies indicate that the Golgi apparatus may also be a Ca^{2+} store in keratinocytes (Hu et al. 2000, Sudbrak et al. 2000). Ca^{2+} is accumulated into intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5-trisphosphate (IP_3) via IP_3 receptors and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (Clapham 1995a and b, Putney 1999). A connection has been demonstrated between the filling status of the intracellular calcium stores and the plasma membrane calcium channel activity (Putney 1986 and 1990). Extracellular Ca^{2+} enters the cell through various types of plasma-membrane Ca^{2+} channels. Soluble proteins, such as calmodulin, contribute to the buffering of cell Ca^{2+}, but membrane-intrinsic transporting proteins are more important. Ca^{2+} is transported across the plasma membrane (channel, pump, Na^+/Ca^{2+} exchanger) and across the membranes of organelles.

External signals arriving at the cell engage plasma membrane receptors to initiate cell signaling pathways. One of the end results is increased intracellular calcium concentration. On stimulation this level can rise globally to in excess of 1 M. This increase can be generated from sources both within and outside the cell. The formation of IP_3 is the focal point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly. These separate receptor mechanisms are coupled to energy-requiring transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-biphosphate to generate both DAG and IP_3. The latter then binds to an IP_3 receptor (IP_3R) to mobilize stored calcium and to promote an influx of external calcium.

Figure 2. Representation of calcium homeostasis in a single cell. Extracellular Ca^{2+} enters the cell through plasma membrane Ca^{2+} channels and leaves the cell using Ca^{2+} pumps and Na^+/Ca^{2+} exchangers. Endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Ca^{2+} is accumulated in intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5-trisphosphate (IP_3) via IP_3 receptors (IP_3R) and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (RyR). Store-operated calcium channels (SOCs) open in response to depletion of the (ER) Ca^{2+} stores. Calcium influx factor (CIF) has postulated to mediate the signal from IP_3R to the plasma membrane store-operated calcium channels (SOCs).

Many important aspects of cellular physiology are regulated by the free cytosolic Ca^{2+} concentration. The intracellular Ca^{2+} signal is regulated in space, amplitude, and frequency. In both excitable and nonexcitable cells, Ca^{2+} increases have a complex temporal and spatial arrangement (e.g.

oscillations and waves) (Lechleiter *et al.* 1991, Thomas *et al.* 1991). Such global Ca^{2+} signals have been suggested to result from spatially and temporally coordinated recruitment of subcellular Ca^{2+} release units (Bootman & Berridge 1995, Lipp & Niggli 1996). These units represent the elementary building blocks of Ca^{2+} signaling, but the principles underlying their recruitment are largely unknown (Berridge 1997). Examples of elementary Ca^{2+} release events are the " Ca^{2+} sparks" in cardiac muscle cells (Cheng *et al.* 1993, Lipp & Niggli 1994, López- López *et al.* 1995) and the Ca^{2+} puffs in Xenopus oocytes (Yao *et al.* 1995), PC12 cells (Reber & Schindleholz 1996), and HeLa cells (Bootman *et al.* 1997). Such elementary Ca^{2+} release events are highly localized signals of short duration (Bootman & Berridge 1995, Lipp & Niggli 1996), which dissipate rapidly owing to diffusion in the cytoplasm and sequestration into the intracellular stores. Fig. 2. Representation of calcium homeostasis in a single cell. Extracellular Ca^{2+} enters the cell through plasma membrane Ca^{2+} channels and leaves the cell using Ca^{2+} pumps and Na^{+}/Ca^{2+} exchangers. Endoplasmic reticulum (ER) is a major site for sequestered Ca^{2+} ions. Ca^{2+} is accumulated in intracellular stores by means of Ca^{2+} pumps and released by inositol 1,4,5-trisphosphate (IP_3) via IP_3 receptors (IP_3R) and by cyclic adenosine diphosphate ribose (cADPr) via ryanodine receptors (RyR). Store-operated calcium channels (SOCs) open in response to depletion of the (ER) Ca^{2+} stores. Calcium influx factor (CIF) has postulated to mediate the signal from IP_3R to the plasma membrane store-operated calcium channels (SOCs).

Elementary Ca^{2+} signals seem to have two functions. They can either activate processes in the Ca^{2+} microdomain in the immediate vicinity of the Ca^{2+} channel or, by recruiting other channels throughout the cell, they can activate processes through a global increase in $[Ca^{2+}]_i$. To avoid the toxic nature of Ca^{2+} cells use either low-amplitude Ca^{2+} signals or, more usually, transient Ca^{2+} signals, known as Ca^{2+} oscillations. Cells use either amplitude modulation or frequency modulation to decode information from Ca^{2+} signals. For example, the frequency of Ca^{2+} oscillations is used to optimize gene expression. To use frequency modulation based signaling, cells have developed decoders that respond to the frequency and duration of the Ca^{2+} signal, the best known example being calmodulin-dependent protein kinase II (De Koninck & Schulman 1998). One molecule that is sensitive to changes in intracellular calcium levels is Ras. The small GTPase operates as a binary molecular switch, and regulates cell proliferation and diferentation. The release of internally stored Ca^{2+} can lead to Ras activation (Rosen *et al.* 1994, Lockyer *et al.* 2001). In most cases this has been achieved through the addition of reagents such as thapsigargin and ionomycin, which do not release Ca^{2+} with the same spatio-temporal patterning as physiological stimuli. The plasma membrane has been considered to be the crucial location for Ras regulation, but new data is emerging to indicate that endomembrane Ras might also be important for compartmentalized Ras-signaling (Chiu *et al.* 2002). Complex Ca^{2+} signals might influence the spatio-temporal control of Ras activation-deactivation (Cullen & Lockyer 2002).

2.3.3. Regulation of intracellular calcium

$[Ca^{2+}]_i$ increases can be generated from sources both within and outside the cell: Ca^{2+} release from intracellular calcium stores and secondly Ca^{2+} influx across the plasma-membrane. The regulated entry of Ca^{2+} across the plasma membrane is an essential and ubiquitous signaling mechanism in both electrically excitable and nonexcitable cells.

Keratinocytes are a special case concerning the relationship between extracelluler calcium concentration and cellular differentation. Previous studies have shown that changes in the concentration of extracellular calcium affect the balance between proliferation and differentiation in epidermal keratinocytes (Yuspa *et al.* 1989, Menon *et al.* 1992). Specifically, extracellular Ca^{2+} leads to a rapid increase in $[Ca^{2+}]_i$ and IP_3 production as a result of activation of the Ca^{2+}-sensing receptor. Extracellular calcium can thus influence intracellular calcium levels. Elevation of $[Ca^{2+}]_e$ above 0.03 mM (calcium switch) inhibits proliferation and induces the onset of terminal differentiation. One early response to the

elevation of extracellular calcium is an increase in $[Ca^{2+}]_i$ (Sharpe *et al.* 1989). Blocking of the rise in $[Ca^{2+}]_i$ with an intracellular calcium chelator blocks the ability of extracellular calcium to induce differentiation (Li *et al.* 1995). After the calcium switch, the levels of IP_3 and DAG also increase rapidly (Jaken & Yuspa 1988, Bikle & Pillai 1993). This is subsequently followed by elevated expression of differentiation-related genes, such as involucrin (Rice & Green 1979) and transglutaminase (Thacher & Rice 1985, Gibson *et al.* 1996), a substrate and an enzyme, respectively, required for cornified envelope formation. Previous studies (Filvaroff *et al.* 1994, Bikle *et al.* 1996, Tu *et al.* 1999) suggest the involvement of the extracellular calcium-sensing receptor (CaR) in mediating calcium signaling during keratinocyte differentiation. Activation of CaR with calcium or other polyvalent cations activates the phospholipase C-signaling pathway, resulting in the generation of inositol 1,4,5-trisphosphate and the release of calcium from intracellular stores (Garrett *et al.* 1995, Chattopadhyay *et al.* 1996). Human keratinocytes express full-length CaR and an alternatively spliced forms of CaR lacking exon 5 (AltCaR) (Oda *et al.* 1998). Unlike the full-length CaR, AltCaR fails to mediate the acute IP_3 response to $[Ca^{2+}]_e$. The full-length CaR message is maximally expressed in undifferentiated keratinocytes, but its level decreases as the cells differentiate. On the other hand, the message levels of AltCaR remain relatively unchanged throughout differentiation (Oda et al. 1998). These changes in CaR expression are consistent with the reduction in the $[Ca^{2+}]_i$ and IP_3 responses to $[Ca^{2+}]_e$ during differentiation and further support the role for CaR in keratinocyte differentiation (Oda et al. 1998).

Ca^{2+} signaling in electrically excitable and nonexcitable cells differs in a fundamental way: whereas excitable cells in many cases use voltage-gated Ca^{2+} channels to regulate Ca^{2+} entry, nonexcitable cells rely on voltage-independent Ca^{2+} channels. Several classes of voltage-independent Ca^{2+} channels have been discovered, including receptor-operated, ligand-operated, G protein-coupled, and store-operated channels (reviewed by Penner *et al.* 1993, Fasolato *et al.* 1994, Felder *et al.* 1994). Store-operated channels (SOCs), defined as channels that open in response to depletion of the endoplasmic reticulum (ER) Ca^{2+} stores, represent one of the most ubiquitous mechanisms for triggering Ca^{2+} influx in nonexcitable cells. Hypothalamic peptides called orexins (Sakurai *et al.* 1998) or hypocretins (de Lecea *et al.* 1998) mediate their effects through G protein-coupled receptors called OX_1 and OX_2 receptors (Sakurai *et al.* 1998). A previous study has shown that the orexin OX_1 receptor activates the Ca^{2+} influx pathway necessary for coupling to phospholipase C in Chinese hamster ovary cells (Lund *et al.* 2000). On the other hand, PLC- is also activated by PI3 kinase-induced formation of Ptd Ins (3,4,5) P_3, which serves as a binding target for the PH-domain of PLC (Falasca et al. 1998). One of the end results is the activation of two receptor-mediated pathways for stimulating the formation of IP_3 and DAG and the subsequent elevation of $[Ca^{2+}]_i$. The regulation of Ca^{2+} in the nucleus, where some important Ca^{2+}-sensitive processes reside, is a debated issue (Bootman et al. 2000). Finally, if the control of cellular Ca^{2+} homeostasis somehow fails (excess penetration), mitochondria "buy time" by precipitating inside Ca^{2+} and phosphate. If the injury persists, Ca^{2+}-induced cell death eventually ensues (Bootman et al. 2000).

Kinase

Figure 3. Summary of the two major receptor-mediated pathways for stimulating the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). Many agonists bind to 7-membrane spanning receptors (R), which use a GTP-binding protein (G) to activate phospholipase C-1 (PLC-1), whereas PLC-1 is stimulated by the tyrosine kinase-linked receptors. The latter activate other effectors, such as the phosphatidylinositol 3-OH kinase (PI-3K), which generates the putative lipid messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3) and the GTPase-activating protein (GAP) that regulates Ras. IP_3R= IP_3 receptor, PKC=protein kinase C, TRH= thyrotropin-releasing hormone, GnRH=gonadotrophin-releasing hormone, PTH=parathyroid hormone. (The figure has been modified from Berridge 1993).

Over the past several years, electrophysical and molecular biological studies have demonstrated the existence of a diverse family of store-operated channels. The best-characterized SOCs have many salient features. The essential defining feature of these channels is activation by a variety of stimuli that deplete stores, independently of the Ca^{2+} released during the depletion process. These stimuli include agonists to phosphoinositide-linked receptors, intracellular IP_3, Ca^{2+} ionophores, inhibitors of SERCA-type Ca^{2+} ATPases, and intracellular dialysis with buffered solutions containing low concentrations of Ca^{2+} (<50 nM). Because the content of intracellular Ca^{2+} stores it is still difficult to measure and correlate with inflow activation, the identification of a current as being store-operated relies entirely on indirect inference. For this reason, it is essential to test as many methods of store depletion as possible. Activation by IP_3 suggests that the agonist receptor is not directly coupled to channel activation. Activation by ionophores and SERCA inhibitors, which deplete stores without generating IP_3 implies that the channels respond to depletion rather than directly to IP_3. However, because most of these agents increase the net Ca^{2+} flux from stores, it is also important to demonstrate current activation under conditions of constant $[Ca^{2+}]_i$. For example, powerful evidence for a store-dependent mechanism is obtained from current activation by intracellular Ca^{2+} chelators, which are thought to deplete stores by reducing $[Ca^{2+}]_i$ and interrupting a futile cycle of ongoing Ca^{2+} leak and reuptake by stores. These activation criteria have been met for a number of store-operated channels. A second feature common to all SOCs described to date is a lack of voltage-dependent gating, although this need not be a distinctive feature. Despite the lack of voltage-dependent gating, the membrane potential, nevertheless, plays several important roles in regulating the Ca^{2+} influx through SOCs. First, depolarization inhibits Ca^{2+} entry by reducing the driving force for Ca^{2+} entry. Second, hyperpolarization promotes rapid inactivation of Ca^{2+} release-activated Ca^{2+} (CRAC) channels by Ca^{2+}, presumably by raising the local $[Ca^{2+}]_i$ at the inactivation sites (Hoth & Penner 1992 and 1993, Zweifach & Lewis 1996). Hyperpolarization may enhance CRAC channel activity through effects on Ca^{2+}-dependent potentiation (Zweifach & Lewis 1996).

2.3.4. Capacitative calcium influx

The original idea that calcium might enter cells through a capacitative mechanism was first introduced by Jim Putney (Putney 1986). The somewhat surprising hypothesis was that calcium entry was regulated by the filling status of the calcium stores. Analogously to a capacitor in an electrical circuit, the calcium stores prevent entry when they are charged up but begin to promote entry as soon as the stored calcium has been discharged. This capacitative entry mechanism is present in many cells and has properties that are very similar in different cells (Putney 1990, Meldolesi *et al.* 1991, Penner *et al.* 1993, Fasolato *et al.* 1994, Putney & Bird 1994). Capacitative calcium entry can be switched on by a variety of stimuli, such as normal agonists or pharmacological agents, all of which share the property of releasing stored calcium. Examples include calcium-mobilizing agonists (Parker & Miledi 1987, Lupu-Meiri *et al.* 1993, Parekh *et al.* 1993, Zweifach & Lewis 1993, McDonald *et al.* 1993, Petersen & Berridge 1994, Byron & Taylor 1995), the calcium-mobilizing second messenger inositol 1,4,5- triphosphate (IP_3) (McDonald *et al.* 1993, Petersen & Berridge 1994, Hoth & Penner 1992 and 1993, Yao & Parker 1993, DeLisle *et al.* 1995), the calcium ionophore ionomycin (Hoth & Penner 1992, Morgan & Jacob 1994), inhibitors of the endoplasmic reticulum (ER) pumps, such as thapsigargin (Lupu-Meiri *et al.* 1993, Zweifach & Lewis 1993, Petersen & Berridge 1994, Mason *et al.* 1991) and cyclopiazonic acid (Mason *et al.* 1991, Demaurex *et al.* 1992), or simply incubation of cells in Ca^{2+}-free conditions (Petersen & Berridge 1994, Hoth & Penner 1992 and 1993). All this evidence indicates that the entry of external calcium is somehow controlled by the calcium content of the ER. Previous studies have investigated thapsigargin-induced calcium mobilization in keratinocytes (Jones & Sharpe 1994, Harmon *et al.* 1996).

Numerous studies utilizing agents that inhibit the Ca^{2+}-ATPase responsible for Ca^{2+} storage within the endoplasmic reticulum (e.g. thapsigargin) have demonstrated that Ca^{2+} store depletion provides

a full and sufficient signal for the activation of a capacitative calcium influx (Putney, 1993 and 1997). However, the nature of the signal which links the depletion of ER calcium stores to the opening of the capacitative Ca^{2+} channels remains unknown. Several potential mechanisms have been suggested for the coupling of the calcium content of ER and the permeability of the plasma membrane to calcium: Initially, physiological contact between the ER membrane and the plasma membrane calcium channels was proposed (Putney 1986, Irvine 1990). Furthermore, intermediates of IP_3 metabolism, small G proteins, a factor synthesized in calcium-depleted ER, also known as calcium influx factor-hypothesis (CIF-hypothesis), cytochromes P450, tyrosine kinases, protein kinases C and/or protein kinase A, and the cytoskeleton have been suggested to play a role in the coupling between the calcium content of ER and the permeability of the plasma membrane to calcium (Luckhoff & Clapham 1992, Alvarez *et al*. 1992, Parekh *et al*. 1993, Randriamapita & Tsien 1993, Lee *et al*. 1993, Striggow & Bohnensack 1994, Petersen & Berridge 1996, Gregory & Barritt 1996, Holda & Blatter 1997, Ribeiro *et al*. 1997). More specifically, the mechanisms proposed to explain how information is transferred from the endoplasmic reticulum to the plasma membrane fall into two main lines of deduction (for reviews, see Putney & Bird 1993, Berridge 1995b). One model describes a direct protein-protein association between the IP_3 receptor in the endoplasmic reticulum and the capacitative entry channel. In this model, the IP_3 receptor would undergo a conformational change, which would allow its interaction with the capacitative Ca^{2+} channel and its opening (Ribeiro *et al.* 1997). Another possible explanation is that a diffusible messenger is released as a consequence of Ca^{2+} store depletion, which would subsequently stimulate the Ca^{2+} influx channel to open (Holda & Blatter 1997). The first model implies close physical interaction between the endoplasmic reticulum and the plasma membrane mediated by, for example, the cytoskeleton. No direct interaction between the ER and the plasma membrane would be required for a diffusible factor to act. Indirect evidence has suggested that the actin cytoskeleton may mediate physical interaction between IP_3 receptors and the plasma membrane (Rossier *et al*. 1991, Ribeiro *et al*. 1997). This interaction might be important for calcium signaling and for capacitative calcium entry.

Experimental approaches to analyze the signal involved in store-operated calcium entry have been considerably advanced by the discovery of mammalian homologs of the Drosophila cation channel transient receptor potential (TRP). Some members of the TRP family can be expressed to Ca^{2+}-permeable channels that enable SOCs; other members form channels activated independently of stores. TRP proteins may be an essential part of endogenous Ca^{2+} entry channels but so far expression of most TRP cDNAs has not resulted in restitution of channels found in any mammalian cells, suggesting the requirement for further unknown subunits (Zitt *et al.* 2002).

Capacitative calcium entry plays a central role in many aspects of cell signaling. For example, the Ca^{2+} oscillations that have been recorded in many cells are maintained by the small but constant influx of external calcium (Berridge 1990 and 1993). Capacitative calcium influx has also been implicated in the function of osteoclasts (Zaidi *et al.* 1993), the regulation of adenylate cyclase (Cooper *et al.* 1994), regulatory volume decrease (Tinel 1994), phototransduction in *Drosophila* photoreceptors (Hardie 1991, Ranganathan *et al.* 1991, Minke & Selinger 1991, Hardie & Minke 1992, Selinger *et al.* 1993), and the activation of mitogenesis in lymphocytes (McDonald *et al.* 1993) and in fibroblasts (Lee *et al.* 1993). The control of proliferation is a particularly interesting example because certain forms of immunodeficiency may result from a defect in calcium entry (Partiseti *et al.* 1994). Also, there was a marked increase in capacitative calcium entry in simian virus 40 (SV-40)-transformed Swiss 3T3 cells (Newcomb *et al.* 1993). Furthermore, previous studies have shown that an intact actin-containing cytoskeleton is necessary for the activation of store-operated Ca^{2+} channels (Wang *et al.* 2002, Bishara *et al.* 2002, Rafferty *et al.* 1994).

2.3.5. Calcium signaling and disease

Mutations or abnormalities in one of the above mentioned Ca^{2+}-transporting proteins may lead to disease. Two skin diseases are caused by Ca^{2+} pump mutations (Hailey-Hailey disease and Darier disease). Hailey-Hailey disease (HHD, MIM 16960) is inherited in an autosomal dominant manner and characterized by persistent blisters and erosions of the skin (Burge 1992). Hailey-Hailey disease is caused by mutations in the *ATP2C1* gene *e*ncoding a novel Ca^{2+} pump (Sudbrak et al. 2000). The *ATP2C1* gene encodes a new class of P-type Ca^{2+} transport ATPase, which is related to the sarco(endo)plasmic calcium ATPase (SERCA) and the plasma membrane calcium ATPase (PCMA) families of Ca^{2+} pumps. Impaired intercellular adhesion and epidermal blistering also occur in patients with Darier disease (DD, MIM 124200), which is caused by mutations in the *ATP2A2* gene, a gene encoding a sarco/endoplasmic reticulum (ER)-Golgi calcium pump (Richard *et al.* 1990). Recent evidence from Hailey-Hailey disease patients indicates that the Golgi apparatus may also be an important Ca^{2+} store in keratinocytes (Hu et al. 2000, Sudbrak et al. 2000).

Skeletal-muscle pathology may be caused by abnormal ryanodine receptors (malignant hyperthermia, porcine stress syndrome, central core disease), plasma membrane Ca^{2+} channels (hypokalemic periodic paralysis, muscular dysgenesis in mice, paraneoplastic Lambert-Eaton myasthenia syndrome), or Ca^{2+} pumps (Brody disease). Neurologic disorders may be related to altered function of plasma-membrane Ca^{2+} channels (episodic ataxia type 2, spinocerebellar ataxia type 6, familial hemiplegic migraine, glutamate excitotoxicity, tottering, leaner, lethargic and stargazer mice), IP3 receptors (Lowe"s oculocerebrorenal syndrome, manic depression, Alzheimer"s disease, opisthotonos mice) and Ca^{2+} pumps (deafwaddler mouse and wriggle mouse sagami). Incomplete X-linked congenital stationary night blindness is caused by a mutation in the plasma membrane Ca^{2+} channels in rods and cones (for a review see Missiaen *et al.* 2000).

http://herkules.oulu.fi/isbn9514268040/html/i1001924.html

2.4. Intercellular calcium waves

2.4.1. Gap-junctional calcium wave

2.4.1.1. Gap junctions

Gap junctions are intercellular channels, which are involved in cell-cell communication (Burge 1994, Richard 2000). Gap junctions are pervasive intercellular membrane channels that directly connect cells on tissues and organs, allowing inorganic ions and other small water-soluble molecules (<1 kDa) to pass from one cell to another and thereby coupling cells electrically and metabolically (Goodenough *et al.* 1996, Kumar & Gilula 1996). Gap junction biogenesis requires assembly of six subunit proteins, connexin, into a hexameric connexon and their trafficking to the plasma membrane. 14 distinct connexins have been described in rodents, and all appear to have similar arrangement in the membrane (Goodenough *et al.* 1996).

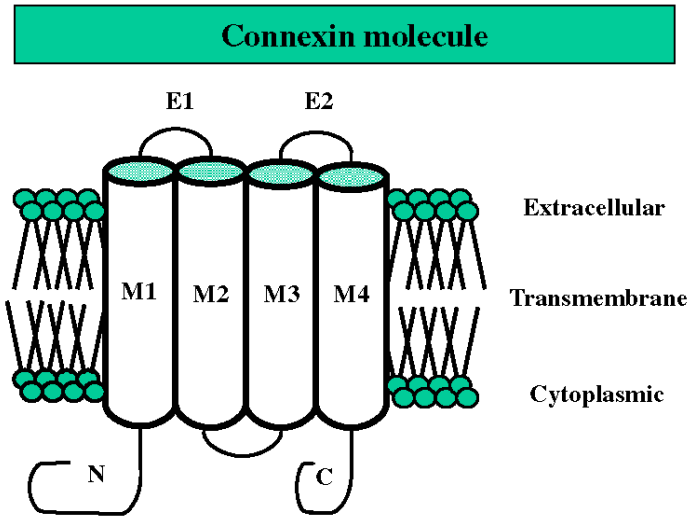

Figure 4. Connexin structure. The cylinders represent transmembrane domains (M1-M4). The loops between the first and second, as well as the third and fourth, transmembrane domains are predicted to be extracellular (E1 and E2, respectively).

Interactions between connexons from jukstaposed cells result in functional gap junction channels. Each gap junction pore is formed by a juxtaposition of two intercellular hemichannels in neighboring cells, which interact to span the plasma membranes of two adjacent cells and directly join the cytoplasm of one cell to that of another. Each hemichannel is composed of a hexameric array of connexins (Goodenough *et al.* 1996, Saez *et al.* 1993). Many different types of connexins can join to form a wide diversity of gap-junctional channels, depending on the tissue and the functional status of the cell (Goliger & Paul 1994, Wiszniewski *et al.* 2000).

Gap junctions have diverse integrative functions, including roles in development, synchronous contraction of the heart, regulation of exocytosis, and control of cell growth (Goodenough *et al*. 1996). Gap junctions are numerous in all layers of the epidermis and have an important role in the coordination of keratinocyte growth and differentiation (Salomon *et al.* 1994, Tada & Hashimoto 1997). Mutations in connexins or defective production of gap junctions are associated with deafness (Kelsell *et al.* 1997), Charot-Marie-Tooth X-linked neuropathy (Bergoffen *et al.* 1993), malignancy (Budunova 1994), and cataractogenesis (Gong *et al.* 1997).

A spectrum of drugs have been shown to inhibit gap-junctional communication with variable degrees of efficacy and specificity. These include volatile anesthetics, such as halothane (Nedegaard 1994), the straight-chain alcohols heptanol and octanol (Donahue *et al.* 1995, Nedegaard 1994), anandamide (Venance *et al.* 1995), and glycerrhetinic acid (Davidson & Baumgarten 1995). Although a variety of drugs have been used to block gap-junctional communication, the specificity of the blockers remains to be solved.

2.4.1.2. Connexins as tumor suppressors

The possible role of GIJC in suppressing the growth of malignant cells was first suggested by Stoker (Stoker 1967). More recent evidence of a direct role of GJIC in tumor suppression has come from a series of experiments in which connexin genes were transfected into GJIC-deficient malignant cell lines, to study the effect of connexins on cell growth *in vitro* and *in vivo*. Thus, human liver tumor cells transfected with the Cx32 gene showed reduced tumour growth (Eghbali *et al.* 1991). Similarly, rat

glioma cells (Naus *et al.* 1992) and chemically transformed mouse fibroblasts (Rose *et al.* 1993) transfected with the Cx43 gene did not produce tumour growth. The ability to suppress tumors is related to specific connexin types. HeLa cells transfected with the Cx26 gene were completely non-tumorigenic and showed a reduced growth rate *in vitro*, while the Cx40 and Cx43 genes did not have a significant tumor-suppressive effect on the same cells, despite an increase in GJIC (Mesnil *et al.* 1994). The Cx26 gene has previously been proposed to be a tumor-suppressor gene of human mammary cancers (Lee *et al.* 1991). The Cx26 gene appears to be the major connexin gene expressed in the cervix. These results suggest that connexin genes exert a differential cell growth control effect, depending on the cell type in which they are expressed.

In certain systems, there is a good correlation between the expression of specific connexin genes and susceptibility to carcinogenesis. For example, the putative tumor suppressor gene, Cx26, is expressed in the liver to different degrees in different species, increasing in the order rat < mouse < guinea pig (Kuraoka & Shibata 1993). The resistence of these rodent species to liver carcinogenesis increases in the same order (Bannasch 1983), suggesting that the extent of Cx26 gene expression is associated with resistance to the induction of liver carcinogenesis.

Previous studies have shown that many different connexins are expressed in human skin. For example, connexin 26 (Cx 26) and connexin 43 (Cx43) are the main connexins in skin. Connexin 26 has been shown to be significantly and connexin 43 slightly upregulated in psoriatic skin *in vivo* (Labarthe *et al.* 1998). It has been suggested that psoriatic keratinocytes compensate for their insufficient need of intercellular communication by increasing connexin 26 channels. Connexin 26 upregulation has also been shown to be a feature of keratinocyte differentiation not only in hyperproliferative skin but also in buccal and vaginal epithelium (Lucke *et al*. 1999). Connexin 43 is widely expressed in different tissues, including brain, heart, kidney, smooth muscle, ovary, and some epithelia, and it is actually the most abundant connexin (Goodenough *et al.* 1996, Musil *et al.* 1990). On cell contact, connexons from neighboring cells may form gap junctions in a matter of minutes or even seconds, suggesting that connexons pre-exist in the plasma membrane (Rook *et al.* 1990).

2.4.1.3. Regulation of gap-junction activity by calcium

Like conventional ion channels, individual gap-junction channels do not remain continuously open; instead, they flip between open and closed states. The permeability of gap junctions is rapidly (within seconds) and reversibly decreased by experimental manipulations that decrease cytosolic pH or increase the cytosolic concentration of free Ca^{2+}. These observations indicate that gap-junction channels are dynamic structures that are gated. They can undergo a reversible conformational change that closes the chanel in response to changes in the cell. When a cell is damaged, its plasma membrane can become leaky. Ions present at high concentration in the extracellular space, such as Ca^{2+}, then move into the cell. The influx of Ca^{2+} into the sick cell causes its gap-junction channels to close immediately, effectively isolating the cell and preventing damage from spreading in this way. Elevated levels of cytoplasmic Ca^{2+} in the vicinity of gap junctions thus result in closure of the channel (Rose & Loewenstein 1975, Crow *et al.* 1994, Lazrak & Peracchia 1993, Unwin & Ennis 1983), but the mechanisms are poorly understood. Such effects may involve soluble factors (Nicholson *et al.* 1998), intermediates, such as calmodulin (Torok *et al.* 1997, Peracchia *et al.* 1996), or possibly accessory adhesion proteins (Jongen *et al.* 1991). The gating of gap junction channels is also regulated by H^+ ions, cAMP, voltage, and phosphorylation by protein kinase C (Goodenough *et al.* 1996). Calcium waves are propagated intercellularly via gap junctions (Boitano *et al.* 1992). Two hypotheses concerning the role of calcium in the operating mechanism of gap junction intercellular channels have been discussed. According to the first, the connexon hemichannels in the plasma membrane, prior to assembly into gap junctions, are in a closed

configuration since they exist in a high (~5 μM) sub-plasma-membrane Ca^{2+} environment. Other studies have shown that the "free" connexon hemichannels in the plasma membrane are closed (Li *et al.* 1996). As these hemichannels accrete and dock with partner channels in juxtaposed cells, they enter into or generate a lower intracellular Ca^{2+} environment at this confined gap junction microdomain, which is more favorable for the opening of the coupled hemichannels. According to the second hypothesis, a uniformly high sub-plasma-membrane Ca^{2+} level exists in cells, including the areas directly beneath gap junctions. Such a relatively high $[Ca^{2+}]_{pm}$ would favor a closed configuration of gap junctions (Rose & Loewenstein 1975). In this scheme, an auxiliary proteins associated with the gap junction is thought to mediate channel opening. The present studies do not distinguish between these two hypothesis, but any mechanism proposed must also take into account other central questions concerning intercellular signaling across gap junctions, especially the way in which Ca^{2+} waves propagate between cells (Boitano *et al.* 1992). With regard to the question of whether connexons are in a closed configuration during their intracellular trafficking, it seems unlikely that the hemichannels are shut by the low ambient cytoplasmic Ca^{2+} levels.

2.4.2. Extracellular ATP-mediated calcium wave

Intercellular calcium waves are stimulus evoked cellular responses in which propagated waves of cytoplasmic Ca^{2+} increases spread from one cell to another. Intercellular calcium waves are considered to serve as a pathway to long-range signaling. The generation of intercellular calcium waves relies partly on the autocrine activity of ATP. Mechanical stimulation of cells results in release of ATP, which activates purinergic receptors on neighboring cells (Osipchuk & Cahalan 1992). Extracellular ATP binds to P2 purinergic receptors, which constitute a large family of receptors that are ion channels (P2X purinoceptors) or couple to G proteins (P2Y purinoceptors). The activation of purinergic receptors, in turn, triggers the release of IP_3 and intracellular calcium stores (Dubyak & el-Moatassim 1993). Suramin and reactive blue are P_{2Y}-purinoceptor antagonists. One P_{2X} antagonist is pyridoxal-phosphate-6-azophenyl-2",4"-disulfonic acid tetrasodium (PPADS). An anti-trypanosomal and anti-filarial drug suramin was used to block P_{2Y}-purinergic receptors in the present study (Dunn & Blakeley 1988, Jørgensen *et al.* 1997, Cotrina *et al.* 1998). Suramin also has other effects. It alters phosphoinositide synthesis, inhibits growth factor receptor binding in HT-29 cells (Kopp & Pfeiffer 1990), and inhibits melanoma heparanase and invasion (Nakajima *et al.* 1991).

2.4.3. Cytoskeleton and calcium signaling

Depletion of intracellular Ca^{2+} stores activates a pathway for Ca^{2+} influx across the plasma membrane, which has been termed capacitative Ca^{2+} entry (Putney 1986). Numerous studies utilizing reagents that inhibit the Ca^{2+}-ATPase responsible for Ca^{2+} storage within the endoplasmic reticulum (e.g. thapsigargin) have demonstrated that Ca^{2+} store depletion provides a full and sufficient signal for the activation of capacitative Ca^{2+} entry (Putney 1993 and 1997). However, the nature of the signal linking pool depletion to the opening of the capacitative Ca^{2+} influx pathway remains unknown. Two general mechanisms have been proposed to explain how information is transferred from the endoplasmic reticulum to the plasma membrane (reviewed Putney & Bird 1993, Berridge 1995). One model describes a direct protein-protein association between the IP_3 receptor in the endoplasmic reticulum and the capacitative entry channel; after IP_3 binding and release of stored Ca^{2+}, the IP_3 receptor would undergo a conformational change, which allows for its interaction with the capacitative Ca^{2+} channel, resulting in its opening. According to the other proposal, a diffusible messenger, released and/or formed as a consequence of Ca^{2+} pool depletion, would stimulate the Ca^{2+} influx pathway to open. The first model

implies a close physical interaction between the endoplasmic reticulum and the plasma membrane; on the other hand, such a direct interaction would not be required for a diffusible factor to act. Indirect evidence has earlier been presented by Ribeiro *et al.* 1997 to show that there is physical interaction between the IP_3 receptors and the plasma membrane that involves the actin cytoskeleton, and the authors speculated that this interaction might be important in signaling capacitative calcium entry (Rossier *et al.* 1991).