CHANGING GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN MOUSE EPIDERMAL CELLS DURING TUMORIGENESIS: A STUDY ON UNDERLYING PROCESSES

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Proefschrift
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Subject headings: gap junctional communication, connexin43, E-cadherin, calcium, cAMP.

Front cover: Immunocytochemical staining of the cell adhesion molecule E-cadherin in the membranes of 3PC cells.

1. Het percentage gefosforyleerd connexin43 in epidermale cellen correleert niet met het nivo van intercellulaire communicatie via gap junctions.
Dit proefschrift.

2. Zowel intracellulair calcium als extracellulair calcium kan een rol spelen in de remming van intercellulaire communicatie via gap junctions door tumor promotoren.
Dit proefschrift.

3. In het proces van tumorvorming kunnen veranderingen optreden in de regulatie van intercellulaire communicatie door calmoduline-afhankelijke processen.
Dit proefschrift.

4. Tumor promotoren kunnen naast een rol in de promotiefase van het proces van tumorvorming, ook een rol spelen in de progressiefase van tumorvorming.
Dit proefschrift.

5. De opvatting van Lucas en Wolf waarin een plasmodesma wordt gedefinieerd als 'intercellulair organel' breekt met de traditie dat een organel een subcellulair en cytoplasmatisch gelocaliseerd cellichaam is en is onjuist.
Lucas W.J. and Wolf S. (1993) Trends in Cell Biology 3: 308-315.
Darnell J. et al. (1990) Molecular cell Biology, 2nd ed. Scientific American Books, Freeman, New York.
Esau K. (1977[1960]). Plant Anatomy, 2nd ed. Wiley & Sons, New York.

6. De opvatting dat 'vertakte plasmodesmata' alleen een vorm zijn van 'secundaire plasmodesmata' is onjuist en leidt tot misvattingen met betrekking tot het functioneren van primaire en secundaire plasmodesmata.
Ding B. et al., (1992) Plant Cell 4: 915-928.
Ehlers K. and Kollmann, R. (1996). Planta 199: 126-138.

7. Gezien het belang van het goed functioneren van Mismatch Repair enzymen dienen, bij een toxicologische screening van verbindingen, mede effecten op het functioneren van deze enzymen bestudeerd te worden.
Parsons R. et al. (1993) Cell 75: 1227-1236.

8. In de gangbare opvatting over de oorzaken van een groot aantal ziektes wordt onevenredig veel nadruk gelegd op de genetische component.

9. Een gebrekkige communicatie is mede oorzaak van veel problemen op alle mogelijke biologische integratie nivo's.

10. Daar de fysieke gesteldheid van personen mede bepalend is voor hun geestelijke stabiliteit en incasseringsvermogen, dient in het onderwijsprogramma van onderzoeksscholen voor promovendi mede 240 uur verplicht sporten te zijn opgenomen.

11. De weigering van het adoptierecht voor homofiele paren is een overschatting van de kwaliteit van de traditionele "hoeksteen van de samenleving".
Psychological Perspectives on Lesbian & Gay Mate Experiences. L.D.Garnets & D.C.Kimmel, New York, NY, Columbia University Press 1993, 421-457.

12. Uit milieuoverwegingen dient de kinderbijslag vervangen te worden door een kinderbelasting. Met de opbrengst van deze gelden kan milieu-besparend gedrag (o.a. kinderloosheid van paren) beloond worden.

Stellingen behorende bij het proefschrift "Changing gap junctional intercellular communication in mouse epidermal cells during tumorigenesis: a study on underlying processes"

Léon Jansen, 14 oktober 1996.

Contents

Abbreviations

ANOVA	Analysis of variance
BoP	Benzoyl Peroxide
Ca^{2+}_e	Extracellular calcium
Ca^{2+}_i	Intracellular calcium
CaM	Calmodulin
Ca/CaM-PK II	Calcium/calmodulin-dependent protein kinase II
Cx43	Connexin43
DDT	1,1'-(2,2,2-trichloroethylidene)bis[4-chlorobenzene]
DMBA	7,12-dimethylbenz[*a*]anthracene
DMSO	Dimethylsulfoxide
EGTA-AM	Ethylene glycol-bis(ß-amino-ethyl ether)-n,n,n',n'-tetraacetic acid acetoxy-methyl ester
GJIC	Gap Junctional Intercellular Communication
IBMX	3-isobuthyl-1-methyl-xanthine
ML-7	1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine
PB	Phenobarbital
PKA	Protein kinase A
PKC	Protein kinase C
REML	Residual maximum likelihood
RpcAMP	Rp-Adenosine-3',5'-cyclic-Monophosphate-triethylamine
SDS	Sodium dodecyl sulphate
SpcAMP	Sp-Adenosine-3',5'-cyclic-Monophosphate-triethylamine
Tg	Thapsigargin
TPA	12-*O*-tetradecanoylphorbol-13-acetate
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide

Chapter 1

General Introduction

Framework of this study

The study described in this thesis has been performed within the framework of an European study on the detection and working mechanisms of non-mutagenic carcinogens in a project of Science and Technology for Environmental Protection (STEP-program), partially financed by the Commission of European Communities (CEC). The work of this study has been carried out at the Agrotechnological Research Institute (ATO-DLO), Wageningen, The Netherlands, and at the International Agency for Research on Cancer (IARC), Lyon, France, in cooperation with the Department of Toxicology and the Department of Food Technology, both of the Agricultural University Wageningen, Wageningen, The Netherlands.

The multistage process of tumor formation

The terms initiation and promotion in the process of tumor formation were for the first time defined in the paper of Friedewald and Rous after applying a two-stage technique for the development of skin tumors in rabbits (1). Later on, in 1974, Boutwell et al. hypothesized that various stages could be distinguished within the process of chemical-induced tumor formation. This model for the multistage process of carcinogenesis has been further specified, and shown to be also suitable for the processes involved in hepatocarcinogenesis (3). According to this model, tumor formation starts with the initiation phase, in which one or more stable carcinogen-induced DNA mutations take place. These mutations transform the phenotype of normal cells into preneoplastic (initiated) cells. During the following stage in the process (the promotion phase), initiated cells undergo processes leading to clonal expansion and the formation of premalignant lesions. In the final progression phase, some of these cells develop to malignant phenotypes and secondary tumors will be formed. Several events are possibly involved during the promotion stage, like inhibition of gap junctional intercellular communication (GJIC), selective cell proliferation, and altered cell differentiation. This thesis will focus on processes involved in the inhibition of gap junctional intercellular communication.

Gap junctions

Gap junctions are transmembrane cell specialisations (channels), permitting small molecules (less than 1000 Dalton) to pass to neighbouring cells (4). These channels are often found in (sterol rich) clusters in plasma membranes, called plaques (5). One gap junction consists of two connexons (one per cell), which in turn consists of 6 subunits,

called connexins (6). Several connexins with different molecular weight exist, and the name of the connexins is directly coupled to their molecular weight. For instance, connexins with a molecular weight of 43 kDa are named connexin43 (Cx43). Currently 12 different connexins have been described (7). The expression of the connexins is tissue-dependent and also dependents on the developmental stage of the tissue. More types of connexins can be expressed at a specific points in time in one cell type. The connexons formed by two identical sets of connexins can form functional channels (homologous communication). In addition, gap junctions can also be formed between cells expressing different types of connexins (heterologous communication), although the conductance is often less compared to homologous communication (8).

Evidence for a role of GJIC in the process of tumor formation

Several lines of evidence support a role for (inhibition of) GJIC in the process of tumor formation. First of all, generally the level of GJIC appears to be lower between cancer cells compared to the GJIC level between normal cells in vivo (9,10). Secondly, several agents with tumor promoter capacity in vivo, have been shown to decrease GJIC in several cell types (11,12). Thirdly, expression of several oncogenes can inhibit GJIC (13). In the fourth place, the growth of transformed cells can be inhibited by the stimulation of GJIC between these cells and non-transformed cells (14), and by transfection of transformed cells with a gene coding for a connexin (15). Finally, agents with an inhibitory effect on tumor formation can stimulate GJIC (16,17), and inhibition of GJIC by tumor promoters can be counteracted by these agents (17,18). All these data support the notion that inhibition of GJIC, and changes in the regulation of GJIC, plays a role in the process of tumor formation.

Regulation of GJIC

Theoretically, the level of GJIC can be influenced at different levels. First of all, in principle, the functioning of connexins could be affected by mutations of the connexin gene. However, this has only been reported for Cx32 in humans with the so-called Charcot-Marie-Tooth neuropathy (19). Secondly, connexin levels may be regulated on the transcriptional level, as was shown for the tumor promoter phenobarbital, which decreased the amount of Cx32 mRNA in livers (20). Thirdly, the GJIC level can be regulated by mechanisms controlling the localization of Cx protein. It has been reported by several groups, that treatment of cells with GJIC-inhibiting agents resulted in a decreased immunostaining of connexin on the plasma membrane (21,22). Finally,

the functioning of connexins can be regulated posttranslationally, i.e. by binding of intracellular proteins to connexins (23) or by phosphorylation of connexins (24). Phosphorylation of connexins could change the permeability of the gap junction by a shift in the tertiary structure of the connexins within one connexon. Furthermore, phosphorylation of connexins is supposed to be involved in the assembly of connexins in the plasma membrane (24), although this is not always the case (25).

Posttranslational regulation of connexins could be triggered by different intracellular second messengers, such as Ca^{2+} and cAMP (Fig.1). The extracellular calcium concentration ($[Ca^{2+}]_e$) in mammalian cells is approximately 10^4 times higher compared to the intracellular calcium concentration ($[Ca^{2+}]_i$), which is about 100 nM. Cells maintain their low $[Ca^{2+}]_i$ by pumping Ca^{2+} against the concentration gradient to the extracellular space, or into intracellular calcium stores (26). It has been reported that changes in $[Ca^{2+}]_e$ and $[Ca^{2+}]_i$ can affect the GJIC level in several cell types. The increase of extracellular calcium concentrations from 0.05 mM to 1.20 mM have been reported to stimulate GJIC in mouse papilloma- or carcinoma-derived cell lines up to 6 hours after changing $[Ca^{2+}]_e$ concentration, but not in primary keratinocytes or a cell line consisting of initiated cells (27). In addition, 2 mM Ca^{2+}_e has been shown to decrease GJIC in primary keratinocytes after 72 hours in these conditions (28), whereas a decreased $[Ca^{2+}]_e$ (to 0.05 mM) inhibited GJIC in human keratinocytes (29).

Concerning changes in $[Ca^{2+}]_i$, a decreased GJIC was reported in bovine lens cell cultures (30), in salivary gland cells (31), and in hepatoma cells (32) when the intracellular calcium concentration ($[Ca^{2+}]_i$) was increased. The $[Ca^{2+}]_i$ can be increased 1) by an increased influx over the plasma membrane; 2) by inhibition of the activity of the Ca^{2+} pumps; 3) as a result of specific intracellular signals like binding of inositol 1,4,5-trisphosphate to receptors on intracellular calcium stores (26). Ca^{2+}_i can exert its effect by activation of the calcium-binding molecule calmodulin (CaM), which was shown to bind to connexin proteins (23). Indeed, CaM antagonists have been reported to increase the GJIC level (33). Furthermore, inhibition of GJIC by several tumor promoting agents can be counteracted by inhibition of CaM (34). This could be the result of stimulation of a Ca^{2+}/CaM-dependent protein kinase (Ca^{2+}/CaM-PK), which can phosphorylate connexin proteins (35).

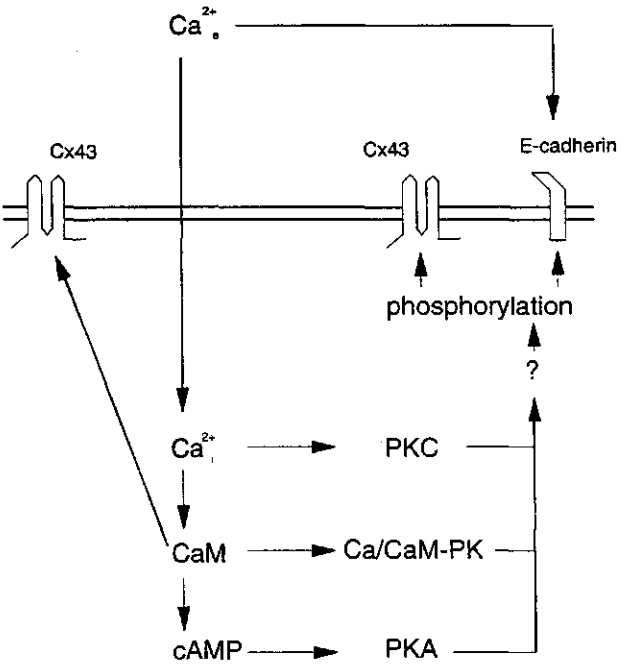

Figure 1: Possible regulation routes of GJIC initiated by a changed $[Ca^{2+}]_e$. See the text for explanation of the figure.

Another intracellular signalling molecule involved in the regulation of GJIC is cAMP. This component is synthesized from ATP by the plasma membrane-bound enzyme adenylate cyclase, and is rapidly degraded by cAMP phosphodiesterases, whose activity is CaM-dependent (36). Increases of the cAMP concentration have been reported to increase GJIC levels in several cell types, on the contrary in other cells it had no effect on GJIC levels (37). The regulation of GJIC by cAMP is most probably due to the activation of cAMP-dependent protein kinase A (PKA), which has been shown to phosphorylate connexins (35). Besides Ca^{2+}/CaM-PK and PKA, evidence for a role of the Ca^{2+}- and lipid-dependent protein kinase C (PKC) in the regulation (inhibition) of GJIC has been shown (38). Finally, GJIC can be regulated by activation of tyrosine kinase. Activation of the src oncogene led to inhibition of GJIC in addition

with phosphorylation of connexin proteins on tyrosine residues (39). All these data support the hypothesis that in the regulation of GJIC several signal transduction routes are involved which lead to changes in phosphorylation levels of gap junction proteins.

GJIC and cell adhesion

Cell adhesion molecules are cell type specific molecules which are integral plasma membrane proteins and facilitate binding to other cells or to the extracellular matrix. The binding of a cell adhesion molecule to the same molecule of an adjacent cell could decrease the intercellular space, hereby facilitating the formation of other intercellular connections like tight junctions or gap junctions.

Several kinds of cell adhesion molecules exist, which are classified into several distinct and structurally diversified families, including integrins, adhesion molecules of the Ig superfamily, LEC-CAMs, cadherins, and others (40). The cadherins are one of the most important molecules in the cell adhesion between epidermal cells. Currently eight types of cadherins can be distinguished: E-cadherin (also known as uvomorulin, L-CAM, arc-1, and cell CAM 120/80), N-cadherin (also known as A-CAM or N-Ca1 CAM), P-cadherin, R-cadherin, EP-cadherin, B-cadherin, T-cadherin, and M-cadherin (41). Cadherins are transmembrane molecules with two extracellular calcium binding sites which are essential for (Ca^{2+}_e-dependent) adhesion to the cadherin molecule of a neighbouring cell (42). How cadherins regulate GJIC is still unknown. Binding to cadherins of the adjacent cell might result in a decreased intercellular space, facilitating the possibility for connexons of neighbouring cells to form functional gap junctions. Furthermore, a cadherin binding-activated signal transduction route as suggested for integrins might also be possible (43). Several proteins were found to bind intracellularly to E-cadherin. These proteins (called catenins) connect E-cadherin to the cytoskeleton and the binding of these proteins has been shown to be crucial for E-cadherin mediated cell adhesion (44). Furthermore, phosphorylation of ß-catenin on tyrosine residues may regulate E-cadherin functioning (45), suggesting a role of phosphorylation in the regulation of GJIC by E-cadherin. Finally, the function of cell adhesion molecules was shown to be regulated by protein kinase C, an enzyme with GJIC decreasing activity (46).

Cell culture assays based on inhibition of GJIC for the detections of agents with tumor promoting capacity

Many tumor promoters have the capacity to decrease the level of GJIC between cells. Therefore, the observation of effects on GJIC could be a useful assay to detect tumor promoting agents. Two types of assays are used mostly for the detection of agents with GJIC inhibiting capacity: 1) metabolic cooperation assays involving exchange of precursors of nucleic acid synthesis between cells, 2) dye transfer assays to measure the passage of a fluorescent dye to adjacent cells. The sensitivity for the detection of agents with tumor promoting capacity in vivo is about 60% for both types of assays (12). Besides these two assays, several other assays have been developed to study effects of tumor promoting agents on a molecular level (Table 1). Mainly, the following types of cells or cell lines are used in these assays: V79 (Chinese hamster lung fibroblasts), 3T3 cells (fibroblasts), mouse or rat hepatocytes, mouse epidermal cell lines or human teratocarcinoma cells. These cells could be less sensitive to effects of tumor promoters on GJIC compared to initiated cells, because of the fact that in the process of tumor formation, initiated cells are the target cells for tumor promoting agents. Furthermore, these cell types could differ in the biotransformation capacity of xenobiotics. Indeed, it is shown that preneoplastic hepatocytes are more sensitive to inhibition of GJIC than primary hepatocytes (63). However, a detailed study on effects of tumor promoters on GJIC in cells representing different stages of tumor formation has not yet been reported.

Objectives and approach of the studies in this thesis

As shown by the study of Jongen et al. (1991), differences exist in the level of GJIC between cells representing different stages of the process of tumor formation. Under low Ca^{2+}_e conditions (0.05 mM), the GJIC level in a carcinoma-derived cell line (CA3/7) was much lower compared to the GJIC levels of a cell line consisting of initiated cells (3PC) or primary keratinocytes. When $[Ca^{2+}]_e$ was increased to 1.20 mM, the GJIC level in CA3/7 cells increased to the same (high) GJIC level of primary keratinocytes and 3PC cells. How a changed $[Ca^{2+}]_e$ affects GJIC is yet unknown. Post-translational regulation of both Cx43 levels and function, and the functioning of E-cadherin might be involved (27). Accordingly, the following model is proposed, as showed in Figure 1. This theory is based on the hypothesis that a changed $[Ca^{2+}]_e$ regulates the GJIC level by changing activities of enzymes involved in intracellular signal transduction routes. The observed differences in the effects of a changed $[Ca^{2+}]_e$

Table 1: Examples of methods used to study inhibition of gap junctional intercellular communication.

Method	Cell type	Reference
Metabolic cooperation	Mouse epidermal cells/ Swiss 3T3 cells	47
	Chick embryo hepatocytes/ Chinese hamster V79 cells	48
	Chinese hamster V79 cells	49
Electric coupling	Human amniotic membrane epithelial cells	50
	BALB/c 3T3 cells	51
Dye transfer		
microinjection in vitro	Human colon epithelial cell line	52
microinjection in vivo	Rat liver	53
photo bleaching	Human teratocarcinoma cells	54
Scrape loading	Several cell types	55
Gap junction structure		
electron microscope	Chinese hamster V79 cells	56
	Mouse skin in vivo	57
	Rat liver in vivo	58
Connexin phosphorylation	Chinese hamster V79 cells	59
Connexin location	Mouse primary keratinocytes in vitro	60
	Rat liver in vivo	61
Connexin mRNA expression	Mouse primary keratinocytes in vitro	60
	Rat liver in vivo	62

on GJIC in two cell types (3PC cells versus CA3/7 cells) may be the result of differences in the responsiveness of enzymes involved in signal transduction routes. As shown in Figure 1, an increased $[Ca^{2+}]_e$ might lead to a higher intracellular calcium concentration, which in turn could activate the calcium binding molecule calmodulin (CaM). Activated calmodulin could change the gap junction functioning by direct binding to connexins, by changing the cAMP concentration and activity of protein kinase A (PKA), or by activation of Ca^{2+}/CaM-dependent protein kinases. These protein kinases might phosphorylate connexins directly or indirectly by (in)activation

of other protein kinases, leading to a changed phosphorylation level of gap junction proteins or (E-cadherin regulating) catenins, and subsequent changed GJIC.

In order to test this hypothesis, the role of molecules and enzymes involved in signal transduction routes in the regulation of GJIC in 3PC cells and in CA3/7 cells was studied and described in chapter 3 (regulation by Ca^{2+}, CaM, and Ca^{2+}/CaM-PK) and chapter 4 (regulation by cAMP and PKA). In these chapters the effects of modulators of Ca^{2+}- or cAMP-dependent processes on GJIC, Cx43 phosphorylation, Cx43 localization and E-cadherin localization are presented.

The second objective of this thesis is to study differences in susceptibility of cell types, representing different stages of tumor formation, for inhibition of GJIC by tumor promoters. In chapter 5 the effects of several known tumor promoters, and of several agents suspected to have tumor promoting activity, on GJIC are described. Because differences in susceptibility of cells for the inhibition of GJIC by tumor promoters may be due to different regulation mechanisms, the effects of tumor promoters on Cx43 phosphorylation, Cx43 localization and E-cadherin localization were studied in primary keratinocytes, 3PC cells and in CA3/7 cells. In the chapters 6 and 7, the results of this study are presented.

Because intracellular calcium plays a role in the regulation of GJIC (chapter 3), the effects of tumor promoters on the intracellular calcium concentration in both 3PC cells and CA3/7 cells were studied and described in chapter 8. In this chapter, also the role of extracellular calcium on tumor promoter-induced changes of GJIC and $[Ca^{2+}]_i$ is described. Finally, the results of the studies described in this thesis are summarized and discussed in chapter 9.

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Chapter 2

Materials and Methods

The following methods were used for the studies described in this thesis:

Materials

For the study on the mechanisms controlling GJIC (chapter 3 and 4), the following chemicals were used: Lucifer yellow CH (LY), dimethyl sulfoxide (DMSO), 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7), N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7), calmidazolium (CDZ), ionomycin, thapsigargin (Tg), forskolin, and 3-isobuthyl-1-methyl-xanthine (IBMX) (obtained from Sigma, St.Louis MO, USA). Ethylene glycol-bis(ß-amino-ethyl ether) n,n,n',n'-tetraacetic acid acetoxy-methyl ester (EGTA-AM) (obtained from Molecular Probes, Eugene, USA). Rp-Adenosine-3',5'-cyclic-Monophosphate-triethylamine (RpcAMP) and Sp-Adenosine-3',5'-cyclic-Monophosphate-triethylamine (SpcAMP) (obtained from Research Biochemicals International, Natick MA, USA). LY was dissolved in 0.33 M LiCl; ML-7, W-7, CDZ, IBMX, EGTA-AM, ionomycin, Tg and Fura2-AM were dissolved in DMSO; and forskolin, EGTA, RpcAMP, and SpcAMP were dissolved in water.

The following agents were used to study the tumor promoter-induced (mechanisms of) GJIC inhibition in cell types representing different stages of tumor formation (chapter 5-9). Benzoyl peroxide (BP), sodium fluoride (NaF), L-ethionone, d-limonene, o-anisidine and phenobarbital (PB) were obtained from Aldrich Chemie, Bornum, Belgium; aroclor 1260 from Interchim, Asnieres, France; 1,1'-(2,2,2-trichloroethylidene)bis[4-chlorobenzene] (DDT)) from Cluzeau info lab, Sainte-Foy-la-Grande, France; and clofibrate, and 12-o-tetradecanoyl-phorbol-13-acetate (TPA) from Sigma, St.Louis MO, USA. The used agents are considered to be tumor promoters due to their non-mutagenicity in S. typhimurium and their carcinogenic effects in in vivo experiments (see chapter 5).

DDT, o-anisidine and TPA were dissolved in dimethyl sulfoxide (DMSO, Sigma); clofibrate and d-limonene in ethanol (Sigma); NaF and PB in distilled water; L-ethionone in 1 M HCl; and aroclor 1260 in acetone (Sigma).

Cell lines and cell culture.

The cell lines used in this study (3PC and CA3/7) represent an early and a late stage of mouse skin carcinogenesis and are described by Klann et al.(1989). In brief: the 3PC cell line was obtained after in vitro initiation with DMBA and selection for calcium resistance. These cells are thought to be initiated cells because of the fact that these

cells do not differentiate in response to high (1.20 mM) extracellular calcium conditions (2). The CA3/7 cell line was obtained from a carcinoma 16 weeks after completion of the DMBA/TPA regimen. The characteristics of these cell lines and the cell culture procedures are described elsewhere (1, 3). The cells were cultured in low calcium medium (0.05 mM). For experiments under high extracellular calcium conditions, cells were cultured for 24 hours in medium containing 1.20 mM Ca^{2+}, after which experiments were performed in the same medium.
HepG2 cells were cultured in F10-medium supplemented with 10% FCS (GIBCO Europe BV, Breda, The Netherlands) under the same conditions as the 3PC cell line. Primary keratinocytes from CD-1 mice were isolated and cultured as described by Klann et all. (1).

Gap junctional intercellular communication (GJIC).
GJIC was measured by the fluorescent dye transfer method, using microinjection of Lucifer Yellow CH and counting the number of fluorescent cells 10 minutes after injection. After determination of the control level, the cells in the same petri-dish were subsequently exposed to the test agent (t=0) and GJIC was determined at different time-points after t=0. At every time-point 20 cells were injected and every experiment was performed at least in duplicate (variation < 7%). Experiments under conditions without Ca^{2+}_e were performed by washing a confluent cell layer with Ca^{2+}-free PBS and subsequent exposure of the cells to the selected agents in PBS. GJIC under high extracellular (Ca^{2+}_e) calcium conditions was determined by culturing the cells for 24 hr in medium containing 1.2 mM calcium. Then the control GJIC was determined, after which the cells were exposed to a test agent under the same Ca^{2+}_e conditions. The control GJIC levels at low respectively high extracellular calcium conditions were for 3PC cells 31.9 and 34.8, and for CA3/7 cells 2.5 and 34.6 (variation <10%). The control GJIC level of primary keratinocytes and HepG2 cells under low extracellular calcium conditions were 18 and 3 respectively. Results are expressed as the percentage remaining GJIC, or as the percentage of GJIC inhibition compared to GJIC at t=0 (100%). Agents that did not show direct inhibition of GJIC in the cell line 3PC (chapter 5) were further tested in two ways: A) after a preincubation of the agent with an aroclor 1254 induced liver homogenate S9 and the appropriate co-factors for 30 minutes (64), cells were exposed to this mixture and GJIC was determined and B) in the cell line HepG2 to determine the possible role of metabolic activation of these agents. At least 40 HepG2 cells were injected and the experiments were performed in

duplicate. Cell viability was tested by Trypan Blue exclusion directly after determination of GJIC and was never below 95%. In the concentrations used, solvents had no effect on GJIC.

A B

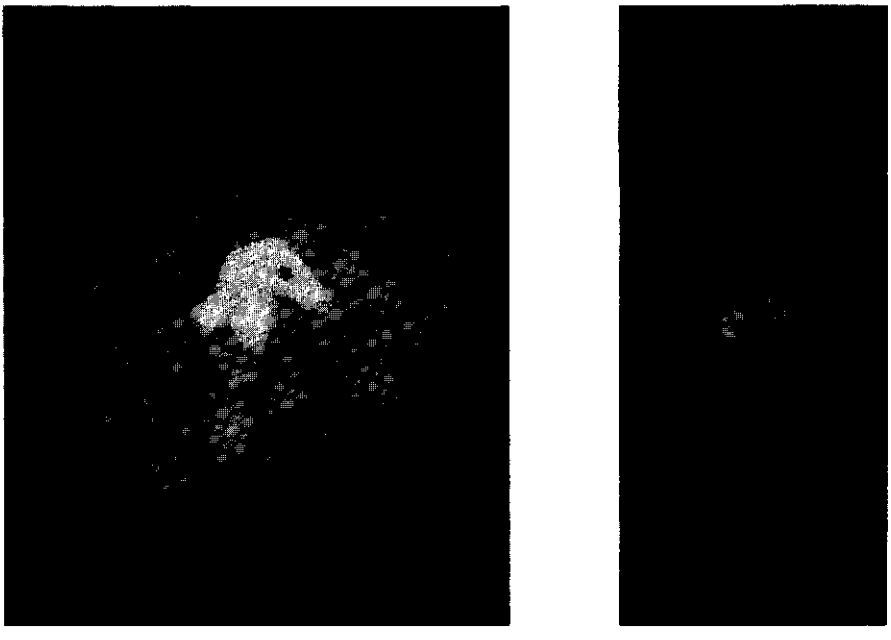

Figure 1: Examples of GJIC in a cell line with a high GJIC level (A) or low GJIC level (B). The cell which is microinjected with the fluorescent dye, is marked (*).

Western blotting.

Directly after GJIC measurements, whole cell protein extract was obtained from the treated cells by cell lysis with a sample buffer (62.5 mM Tris-HCl, pH 6.8, 0.5% SDS). Protein quantification and Western blotting of connexin43 (Cx43) were performed as described by Mesnil et al.(4). Analysis of E-cadherin was done using a 10% polyacrylamide gel and a rat anti E-cadherin primary antibody (Sigma, St Louis, MO). For the determination of both the Cx43 and E-cadherin protein, the ECL-technique of Amersham (Little Chalfont, England) was used. The efficiency of gel electrophoresis and blotting were checked by Coommassie Blue and Ponceau red

staining, respectively. The percentage of phosphorylated Cx43 was determined by scanning the autoradiograms from the Western analysis on the Quantity One system from pdi, Huntington Station, NY. The results are expressed as the percentage phosphorylated CX43 relative to the percentage phosphorylated Cx43 under control conditions (which were under low Ca^{2+}_e conditions 40.6% and 27.9%, and under high Ca^{2+}_e conditions 59.5% and 56.3% for 3PC cells and CA3/7 cells respectively).

Immunocytofluorescence.

For E-cadherin and Cx43 immunostaining, cells were handled as described by Mesnil et al. (4). For E-cadherin staining, the same antibody was used as for Western blotting technique, for Cx43 the mouse derived antibody was purchased from TEBU (Nottingham, UK). Biotinylated second antibodies and FITC-conjugated streptavidin from Sigma (St Louis, MO, USA) were used. The level of immunostaining was determined as 1 of 5 grades of staining, ranging from high to undetectable (+++, ++, +, +/- and -).

Intracellular calcium measurements.

The intracellular calcium concentration was determined by loading the cells with 2,67 μM Fura2-AM (Sigma, St.Louis MO, USA) for 30 minutes at room temperature. The intracellular calcium concentration was determined using the Cue-2 dual wavelength calcium measurement system (PAES bv, Zoeterwoude, The Netherlands) and by measuring the fluorescence ratio by dividing the fluorescence values at wavelengths 340/380. These ratios were transformed into intracellular calcium concentrations by using the formula

$$[Ca^{2+}]_i = Kd * ((R-Rmin)/(Rmax-R)) * (Sf/Sb)$$

in which Kd is the dissociation constant of Fura-2 and Ca^{2+} (224 nM); R is the measured ratio; Rmax is the maximum ratio after addition of KCN, ionomycin and $CaCl_2$ to final concentrations of 4 mM, 10 μM and 1.2 mM, respectively; Rmin is the minimum ratio after addition of 10 mM EGTA; and Sf and Sb are the fluorescence values at 380 nm at Rmin and Rmax calcium concentrations, respectively (5). Rmax and Rmin were determined directly after the experiment. The given intracellular calcium concentrations are the mean of at least 2 experiments, in which the influence of calcium modulating agents on the intracellular calcium concentrations of 10 individual cells was determined.

Statistics.

For each concentration, GJIC or Ca^{2+}_i at different time points were compared to the values for the control using ANOVA with a block effect to allow for systematic differences between petri-dishes. The effect of time and agent were investigated using the REsidual Maximum Likelihood method (REML) of the Genstat 5 package (Genstat, Rothamsted, UK). This method is similar to ANOVA, however it allows for the fact that there are different numbers of cells for each combination of agent and time-point, causing the datasets to be unbalanced in the statistical sense. The variation within one experiment, and between the two duplicate experiments were always below 15% for both GJIC and intracellular calcium experiments.

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Chapter 3

Differences in the calcium-mediated regulation of gap junctional intercellular communication between a cell line consisting of initiated cells and a carcinoma-derived cell line.

This chapter is based on the paper:
L.A.M.Jansen, T.de Vrije and W.M.F.Jongen (1996) Differences in the calcium-mediated regulation of gap junctional intercellular communication between a cell line consisting of initiated cells and a carcinoma-derived cell line. Carcinogenesis, in press.

Abstract

Differences in calcium-mediated regulation of gap junctional intercellular communication (GJIC) between a cell line consisting of mouse epidermal initiated cells (3PC) and a mouse epidermal carcinoma-derived cell line (CA3/7) were studied. Under low extracellular calcium (Ca^{2+}_e) conditions (0.05 mM) CA3/7 cells showed a low level of GJIC compared to 3PC cells. High Ca^{2+}_e (1.20 mM) raised GJIC between CA3/7 cells to the GJIC level of 3PC cells, which in turn remained unchanged under these conditions. Raising the free intracellular calcium concentration (Ca^{2+}_i), using a calcium ionophore (ionomycin) or the Ca^{2+}-ATPase inhibitor thapsigargin under low Ca^{2+}_e conditions, did not affect the GJIC level between 3PC cells, and increased GJIC between CA3/7 cells. Intracellular calcium chelation in 3PC cells under low Ca^{2+}_e conditions by ethylene glycol-bis(ß-amino-ethyl ether) n,n,n',n'-tetra-acetic acid acetoxy-methyl ester (EGTA-AM) decreased GJIC in this cell line. High Ca^{2+}_e conditions protected both cell lines from a decreased GJIC by EGTA-AM exposure. Inhibition of calmodulin (CaM) by calmidazolium (CDZ) or N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) under low Ca^{2+}_e conditions, inhibited GJIC in 3PC cells and increased GJIC in CA3/7 cells. Inhibition of Ca^{2+}/CaM-dependent protein kinase (Ca^{2+}/CaM-PK) by 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) decreased GJIC in both cell lines. Western analysis showed that Cx43 was more phosphorylated in both cell lines in concurrence with different effects on the GJIC level. Under conditions in which GJIC was inhibited, a decreased immunostaining of Cx43 on the plasma membrane was found. The level of immunostaining of the cell adhesion molecule E-cadherin on the plasma membranes of both cell types remained unchanged under conditions in which GJIC was changed by modulaters of $[Ca^{2+}]_i$, CaM activity, or the Ca^{2+}/CaM-PK activity. These results indicate that differences exist between 3PC cells and CA3/7 cells in the GJIC regulation by intracellular calcium and calmodulin.

Introduction

Intercellular communication through gap junctions (GJIC) is involved in many biological processes like metabolic cooperation (1), cell proliferation (2,3) and cell differentiation (4). It has been hypothesized that in the multistep process of tumor formation, a decreased gap junctional intercellular communication of genetically altered (initiated) cells could favorize their clonal expansion (2,5,6).

Gap junctions are intercellular channels, permitting small metabolites, ions and second messengers to pass to neighbouring cells. The channels are made of juxtaposed connexons crossing the membrane of two adjacent cells. Each connexon is formed by six protein subunits called connexins (7,8). How the functioning of connexons is regulated is still largely unknown but phosphorylation of the connexins could play an essential role. Indeed changes in phosphorylation levels of connexins have been reported to correlate with reduced (9,10) or stimulated GJIC (11). These changes can be achieved by (de)activation of protein kinases, however the mechanisms controlling this way of regulation are unknown. Changes in second messenger concentrations like intracellular calcium or cAMP could (de)activate GJIC modulating enzymes. Alterations in these parameters or the responses of enzymes to second messengers could play a part in the presumed role of altered GJIC in the process of tumor formation.

Earlier research showed that during the process of tumor formation GJIC declines in human keratinocyte cell lines (12), and in mouse epidermal cell lines (13). Elevated extracellular calcium concentrations were shown to restore GJIC of papilloma- and carcinoma-derived (CA3/7 cells) mouse epidermal cell lines to levels comparable with primary keratinocytes, while the high GJIC level of a cell line consisting of initiated mouse epidermal cells (3PC) remained unchanged after increasing the extracellular calcium concentration ($[Ca^{2+}]_e$) (14). This suggests that differences exist in the extracellular calcium regulated control of GJIC between the 3PC cell line and the CA3/7 cell line. An increased $[Ca^{2+}]_e$ might lead to a higher intracellular calcium concentration, which in turn could activate the calcium binding molecule calmodulin (CaM) (Fig 1). Activated calmodulin could change the gap junction functioning by direct binding to connexins (15,16), or by activation of Ca^{2+}/CaM-dependent protein kinases. These protein kinases might phosphorylate connexins directly (17) or indirectly by (in)activation of other protein kinases, leading to a changed phosphorylation level of gap junction proteins and a changed GJIC.

To investigate possible differences between initiated (3PC) cells compared to a carcinoma derived cell line (CA3/7) in the mechanisms controlling GJIC, we studied the role of calcium-mediated alterations in the regulation of GJIC in these cell lines. Because in mouse keratinocytes GJIC was shown to be also regulated by the cell adhesion molecule E-cadherin (14), we also studied the role of calcium mediated processes on the amount and location of E-cadherin.

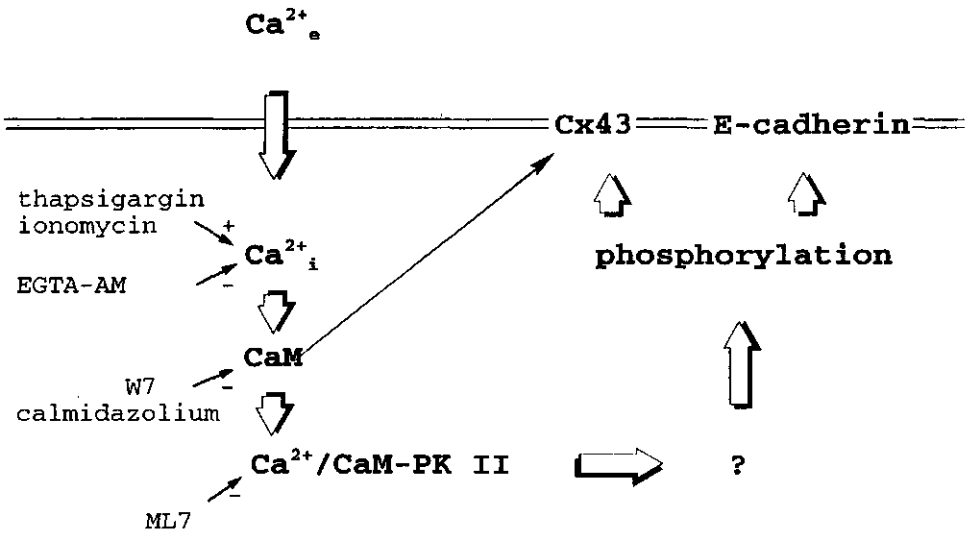

Figure 1: (Working) Hypothesis for the regulation of gap junctional intercellular communication by the calcium signal transduction route. For explanation see introduction. The agents used in this study with a positive (+) or negative (-) effect on a certain concentration or activity are given. Ca^{2+}_e, Ca^{2+}_i= extracellular respectively intracellular calcium; CaM= calmodulin; Ca^{2+}/CaM-PK II= Ca^{2+}/calmodulin dependent protein kinase II; Cx43= connexin43. Small typed agents are used to stimulate (+) or inhibit (-) Ca^{2+}/CaM-dependent processes or concentrations.

Results

Several calcium and calmodulin modulating agents were used to study the calcium dependent regulation of gap junctional intercellular communication (GJIC) in a cell line of initiated mouse epidermal cells (3PC) and in a mouse epidermal carcinoma-derived cell line (CA3/7).

Under low Ca^{2+}_e conditions (0.05 mM), the 3PC cell line has a much higher GJIC level than the CA3/7 cell line (Fig 2A). A high $[Ca^{2+}]_e$ (1.20 mM) did not affect the GJIC

level in 3PC cells, whereas the GJIC level of CA3/7 cells was increased to levels comparable to 3PC cells within 24 hours. At low Ca^{2+}_e conditions, the basal $[Ca^{2+}]_i$ was not significantly different in the 3PC cells compared to the CA3/7 cells (Fig 2B) and ammounted to approximately 110 nM. A change in $[Ca^{2+}]_e$ caused in 3PC cells a first peak in the $[Ca^{2+}]_i$ up to 500 nM after 5 minutes. The $[Ca^{2+}]_i$ returned to the basal level after 7 minutes. A second $[Ca^{2+}]_i$ peak (up to 250 nM) was observed at 90 minutes after the changed $[Ca^{2+}]_e$, and the $[Ca^{2+}]_i$ decreased again to the control level within 24 hours. The Ca^{2+}_e-induced changes in $[Ca^{2+}]_i$, as well as the $[Ca^{2+}]_i$ after 24 hours high Ca^{2+}_e conditions were comparable in 3PC cells compared to CA3/7 cells.

To discriminate between an effect of Ca^{2+}_e or Ca^{2+}_i on GJIC, the $[Ca^{2+}]_i$ was elevated under low Ca^{2+}_e conditions using the calcium ionophore ionomycin (21), or to the Ca^{2+}-ATPase inhibitor thapsigargin (Tg, 22). During the first 90 minutes, the effect of 10 μM ionomycin and 5 μM Tg on $[Ca^{2+}]_i$ in 3PC cells was comparable to the effect of the high Ca^{2+}_e condition on $[Ca^{2+}]_i$ (Fig 3A). Both agents induced a first peak of $[Ca^{2+}]_i$ (up to 370 nM) after which $[Ca^{2+}]_i$ returned to the basal level. In ionomycin exposed 3PC cells $[Ca^{2+}]_i$ increased then up to 200 nM after 90 minutes, whereas in Tg exposed cells $[Ca^{2+}]_i$ remained constant.

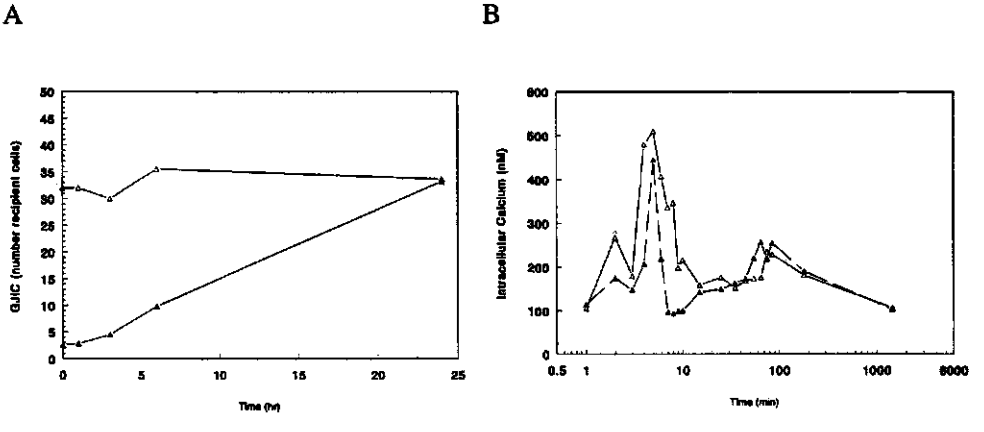

Figure 2: The effect of 1.20 mM Ca^{2+}_e on GJIC (A) and on $[Ca^{2+}]_i$ (B) in 3PC cells and in CA3/7 cells. At t=0 min $[Ca^{2+}]_e$ was increased and the number of communicating cells (A) and the $[Ca^{2+}]_i$ were followed in time. Open symbols: 3PC cells, closed symbols: CA3/7 cells.

A B

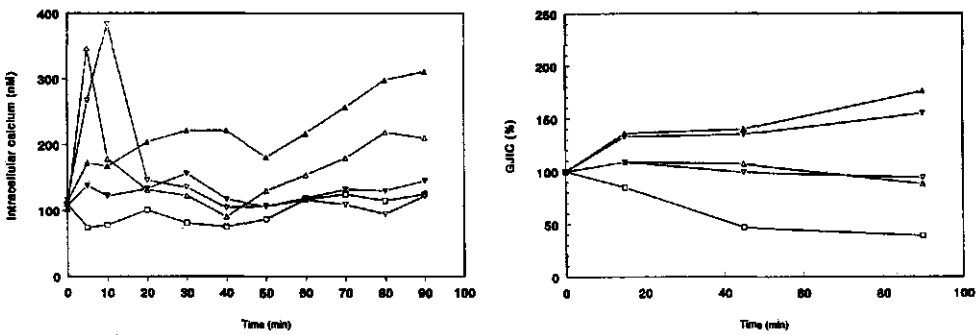

Figure 3: The effect of Ca^{2+}_i modulators on $[Ca^{2+}]_i$ (A) and on GJIC (B). At t=0 min. a calcium modulator was added to the medium and the $[Ca^{2+}]_i$ (A) or the percentage GJIC compared to t=0 (B) were determined. Symbols represent: ▵ 10 μM ionomycin; ▿ 5 μM thapsigargin; □ 100 μM EGTA-AM; open symbols 3PC cells, and closed symbols CA3/7 cells.

In CA3/7 cells however, Tg only slightly increased $[Ca^{2+}]_i$ (150 nM), and ionomycin raised the $[Ca^{2+}]_i$ after 90 minutes to the same level as the high Ca^{2+}_e condition did, however without a first transient peak of $[Ca^{2+}]_i$ at 5-10 minutes. Both ionomycin and Tg had no effect on GJIC in 3PC cells, whereas they increased GJIC in the CA3/7 cell line (Figure 3B). Potentially, a decreased $[Ca^{2+}]_i$ could inhibit GJIC in the 3PC cells (which have a high GJIC level under low Ca^{2+}_e conditions). Therefore both cell types were exposed to the intracellular calcium chelator EGTA-AM (23). As shown in Fig.3A, exposure to 100 μM EGTA-AM decreased $[Ca^{2+}]_i$ only transiently in 3PC cells under low Ca^{2+}_e conditions. In these cells however, the GJIC level was decreased strongly (Fig 3B). Under high Ca^{2+}_e conditions in both cell lines, both GJIC and $[Ca^{2+}]_i$ levels were not significantly changed by EGTA-AM (data not shown).

The changes in $[Ca^{2+}]_i$ could exert their effect on GJIC by changing the activity of calmodulin (CaM) and/or a Ca^{2+}/CaM-dependent protein kinase (Ca^{2+}/CaM-PK). To establish whether differences in GJIC regulation exist between 3PC cells and CA3/7 cells on this level of regulation, the effect of the CaM inhibitors W7 (24) and CDZ (25), and of an inhibitor of Ca^{2+}/CaM-PK (ML-7, 26) on GJIC were studied. In the cell line 3PC, 20 μM W7 decreased GJIC by 50% while in the CA3/7 cell line the level of GJIC was doubled in the same time (Figure 4A).

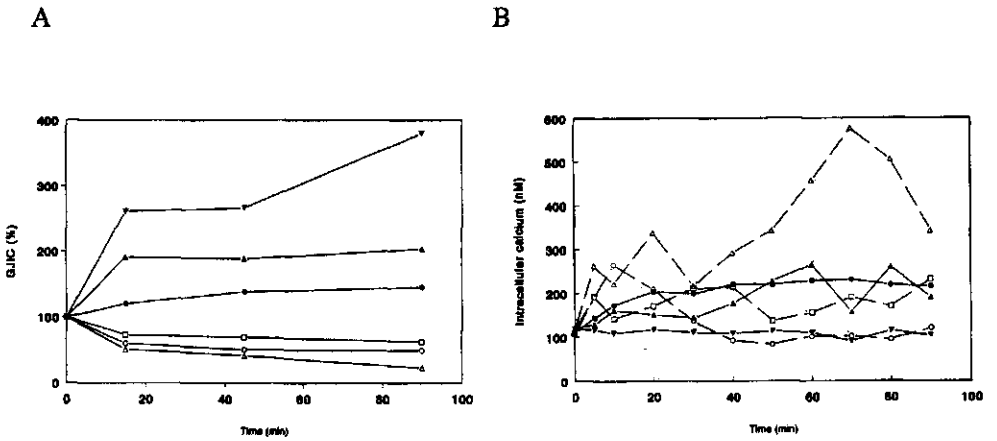

Figure 4: The effect of CaM inhibitors on GJIC (A) and $[Ca^{2+}]_i$ (B). At t=0 min. a calmodulin inhibitor was added to the medium and GJIC (A) or $[Ca^{2+}]_i$ (B) were determined. Symbols represent: ▵ 5 μM CDZ; ▽ 10 μM CDZ; □ 1 μM CDZ; ○ 20 μM W7; open symbols 3PC cells, and closed symbols CA3/7 cells.

CDZ had the same effect as W7 on GJIC in 3PC cells and CA3/7 cells, but it was more effective at lower concentrations. The increase of the GJIC level in CA3/7 cells was faster than the high Ca^{2+}_e-induced increase of the GJIC level. Additionally the effects of these agents on $[Ca^{2+}]_i$ levels were studied. Both agents increased $[Ca^{2+}]_i$ under low Ca^{2+}_e conditions, although the W7-induced increase of $[Ca^{2+}]_i$ was only temporary in 3PC cells (Fig 4B). Whereas 5 μM CDZ increased $[Ca^{2+}]_i$ up to 260 nM in CA3/7 cells after 60 minutes of exposure, 10 μM CDZ had no effect on $[Ca^{2+}]_i$ in CA3/7 cells under these conditions. The Ca^{2+}/CaM-PK inhibitor ML-7 inhibited GJIC in both cell lines in concurrence with a slight increase of the $[Ca^{2+}]_i$ level (Fig 5).

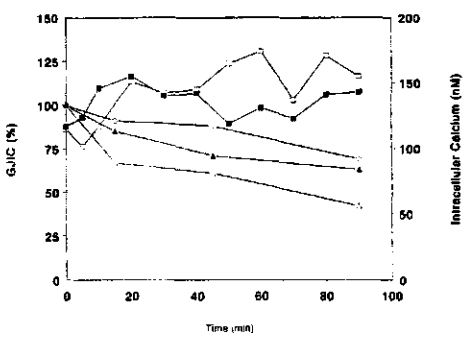

Figure 5: The effect of ML-7 on GJIC and $[Ca^{2+}]_i$. ML-7 was added to the medium at t=0 min. and GJIC or $[Ca^{2+}]_i$ were determined. Symbols represent: ○ 1.0 μM ML-7-GJIC; ▵ 10 μM ML-7-GJIC; □ 10 μM ML-7-Ca^{2+}_i;open symbols 3PC cells, and closed symbols CA3/7 cells.

	3PC		CA3/7	
Calcium	LOW	HIGH	LOW	HIGH
Ionomycin (10 μM)	1.63	nt	2.06	nt
EGTA-AM (100 μM)	1.75	0.92	1.11	1.61
CDZ (5 μM)	1.24	1.06	1.44	1.29
ML-7 (10 μM)	1.79	1.32	1.30	1.52

Table 1: The ratio of the percentage phosphorylated Cx43 in exposed cells and the percentage phosphorylated Cx43 under control conditions. The $[Ca^{2+}]_e$ was 0.05 mM and 1.20 mM for low and high calcium medium respectively. The control % of phosphorylated Cx43 were under low Ca^{2+}_e conditions 40.6% and 27.9%, and under high Ca^{2+}_e conditions 59.5% and 56.3% for 3PC cells and CA3/7 cells respectively. nt= not tested.

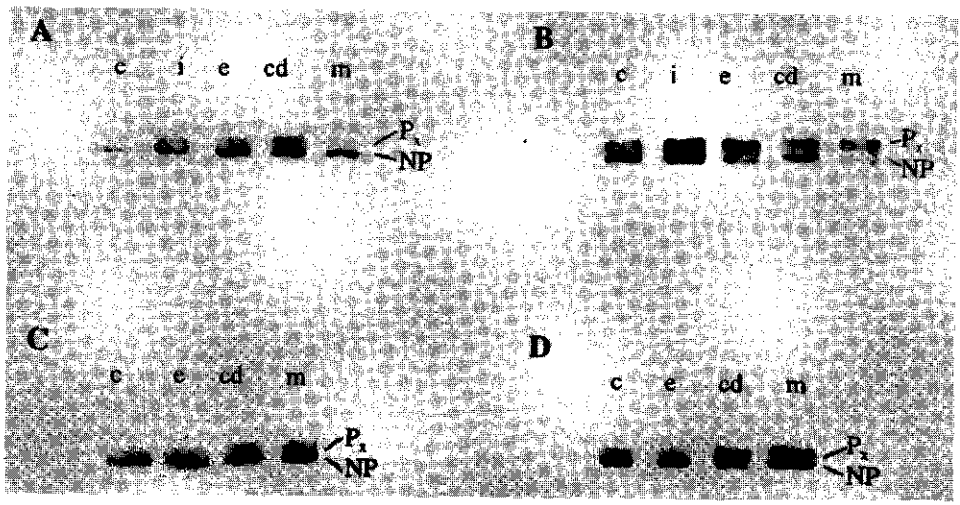

Figure 6: Western blot analysis of connexin43 in CA3/7 cells (A and C) and in 3PC cells (B and D) after 90 minutes of exposure under low (A and B) and high (C and D) extracellular calcium conditions. c=control (untreated) cells, i= 10 μM ionomycin; e= 100 μM EGTA-AM; cd= 5μM CDZ; and m= 10 μM ML-7.

In both cell lines, the effects of ML-7 on both GJIC and $[Ca^{2+}]_i$ were the same under high Ca^{2+}_e conditions as under low Ca^{2+}_e conditions (data not shown). Because the functionality

AGENT (μM)	Calcium (mM)	3PC GJIC[1]	3PC Cx43[2] phosphorylation	3PC Cx43 location[3]	CA3/7 GJIC[1]	CA3/7 Cx43[2] phosphorylation	CA3/7 Cx43 location[3]
Control	0.05	100%	control	+++	100%	control	++
Control	1.20	=	control	+++	↑	control	+++
Ionomycin (10)	0.05	=	↑	=	↑	↑	=
EGTA-AM (100)	0.05	↓	↑	↓↓	=	↑	=
EGTA-AM (100)	1.20	=	=	↓	=	=	↓
CDZ (5)	0.05	↓	↑	↓	↑	↑	=
CDZ (5)	1.20	=	↑	↓	=	↑	↓
ML-7 (10)	0.05	↓	↑	↓↓	↓	↑	↓↓
ML-7 (10)	1.20	↓	↑	↓+c	↓	↑	↓

Table 2: Effect of several agents on GJIC, Cx43-phosphorylation and -immunstaining, and on E-cadherin immunostaining in the cell lines 3PC and CA3/7. 1: Compared to control-L (= 100%) Symbols =,↑,↓ represent an unchanged, an increased or a decreased GJIC respectively; 2: Compared to the control under the same Ca^{++} conditions, Symbols =,↑,↓ represent an unchanged, an increased or a decreased Cx43-phosphorylation respectively; 3: Compared to the control under the same Ca^{++} conditions, Symbols =, ↓, ↓↓, +c represent an unchanged, a decreased, a strongly decreased, or an increased cytosolic intensity of immunostaining respectively.

of gap junctions may be regulated by phosphorylation of the gap junction proteins, the effects of the used calcium/calmodulin modulators on Cx43 phosphory-lation were studied (Fig.6 and Table 1).

Under low Ca^{2+}_e conditions Cx43 is more phosphorylated in the 3PC cells (41%) compared to the CA3/7 cells (28%), under high Ca^{2+}_e conditions the level of Cx43 phosphorylation in both cell lines (respectively 60% and 56%) is comparable (Fig.6). Cx43 phosphorylation was increased in both cell lines under low Ca^{2+}_e conditions by all tested agents (as shown by an increased intensity of the P_x-band in concurrence with a decreased intensity of the NP-band as compared with the control sample). Under high Ca^{2+}_e conditions, both CDZ and ML-7 increased Cx43 phosphorylation in both cell lines, whereas EGTA-AM had no effect on the level of Cx43 phosphorylation in both CA3/7 and 3PC cells. The effects of the used agents on Cx43 phosphorylation in both cell lines are summerized in Tables 1 and 2.

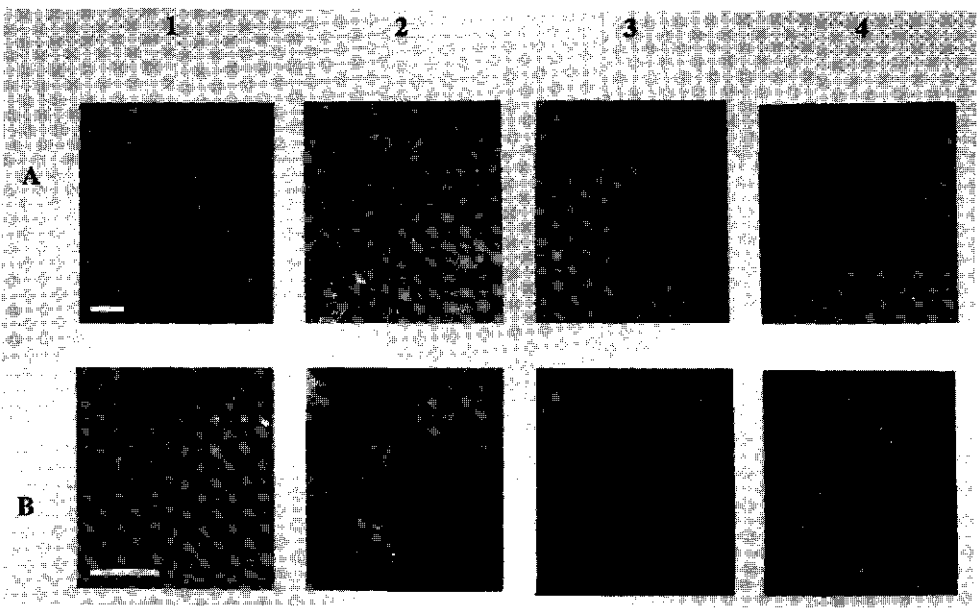

Figure 7: Immunostaining of Cx43 in 3PC cells (A) and in CA3/7 cells (B). Representive pictures are shown of cells under low (A1-A4, B1, B2) or high (B3, B4) Ca^{2+}_e conditions; control staining (A1, B1, B3); a decreased staining (↓ in Table 2, pictures A2, B4); a strongly decreased staining (↓↓, A3, B2), and a decreased staining on the membrane with cytosolic staining (↓+c, A4). Narrow arrow heads mark examples of staining on the membranes, broad arrow head marks an example of cytosolic immunostaining (A4). In addition to the specific staining (examples marked by arrow heads), non-specific autofluorescence of the cells, due to the long exposure time of the films, can be seen. Bar = 25 µm in both series A and B.

Besides a change of the phosphorylation of Cx43, the level of GJIC may be changed by a change in the localization of Cx43. Table 2 and Figure 7 show that under low Ca^{2+}_e conditions, the intensity of Cx43 immunostaining on the membranes of CA3/7 cells was lower than in 3PC cells. Under high Ca^{2+}_e conditions, the immunofluorescence intensity was increased in CA3/7 cells up to a level comparable to 3PC cells. The Cx43-linked immunostaining intensity in 3PC cells was not changed by an increased $[Ca^{2+}]_e$. In both cell lines under low Ca^{2+}_e conditions, ionomycin

exposure did not affect the Cx43-linked immunofluorescence intensity. The EGTA-AM-induced decrease in Cx43-linked immunostaining in 3PC cells was higher under low Ca^{2+}_e conditions compared to high Ca^{2+}_e conditions. In CA3/7 cells EGTA-AM only decreased Cx43 immunostaining under high Ca^{2+}_e conditions. CDZ decreased Cx43 immunostaining in 3PC cells under both Ca^{2+}_e conditions to the same extent. In CA3/7 cells only under high Ca^{2+}_e conditions CDZ decreased Cx43 immunostaining. Under both Ca^{2+}_e conditions, ML-7 decreased Cx43 immunostaining in both cell types.

Because in mouse keratinocytes GJIC was shown to be also regulated by the cell adhesion molecule E-cadherin (14), we studied the effects of mediators of Ca^{2+}-regulated processes on the amount and location of E-cadherin. Neither an increased $[Ca^{2+}]_e$, nor treatment of both cell types with ionomycin, CDZ, or ML-7, changed the amount of E-cadherin in both cell types as determined by Western analysis (as shown for CA3/7 cells in Fig 8A). Changing $[Ca^{2+}]_e$ from 0.05 mM to 1.2 mM strongly increased the immunostaining of E-cadherinon the cell-cell contact areas in

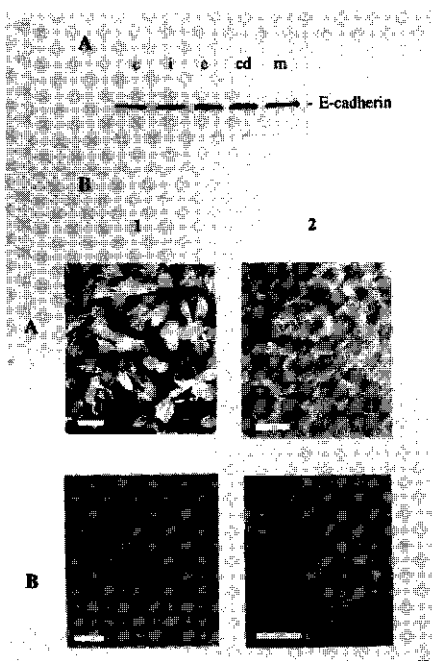

Figure 8: Western analysis (A) and immunostaining (B) of E-cadherin in 3PC cells and CA3/7 cells. A: The amount of E-cadherin remains unchanged after treatment of 3PC cells with modulators of Ca^{2+} dependent processes. Symbols as in Fig.6. This blot is representive for the effect of the used agents on both cell lines under both Ca^{2+}_e conditions. B: E-cadherin immunostaining of 3PC (A) and CA3/7 (B) cells under low (1) and high (2) Ca^{2+}_e conditions. In addition to the specific staining on the membranes (examples marked by arrow heads), non-specific autofluorescence of the cells, due to the long exposure time of the films, can be seen. Bar = 25 µm.

both cell lines (Table 2 & Figure 8B). The intensity of E-cadherin-linked immunofluorescence was not changed by ionomycin, CDZ, EGTA-AM or ML-7 under both low and high Ca^{2+}_e conditions (Table 2).

Discussion

In this study we demonstrate, that a difference in the regulation of GJIC by calcium- and calmodulin-dependent mechanisms between a cell line of initiated mouse keratinocytes and a carcinoma derived cell line exists. Furthermore the data show that changes in GJIC induced by modulators of the calcium signal transduction route, are not associated with concurrent changes in Cx43 phosphorylation levels. Under conditions in which GJIC was inhibited, a decreased level of immunostaining of Cx43 on the membranes was found. The level of immunostaining of the cell adhesion molecule E-cadherin was, under the experimental conditions used in this study, not changed by modulators of Ca^{2+}_i or CaM.

High extracellular calcium conditions did not affect the high GJIC level in 3PC cells. In the mouse skin carcinoma derived cell line however, a shift to 1.20 mM Ca^{2+}_e raised the level of GJIC to that of the 3PC cell line. These results confirm earlier findings (14). Several other groups have reported different effects of Ca^{2+}_e on GJIC. In primary keratinocytes 2 mM Ca^{2+}_e induced a decreased GJIC (27), whereas decreased extracellular calcium conditions (1.8 mM to 0.05 mM) inhibited GJIC in human keratinocytes (12). In this study no differences were found between the effects of high extracellular calcium on $[Ca^{+}]_i$ in 3PC cells and in CA3/7 cells. This suggests that the regulation of calcium transport across the membranes is functioning with a comparable efficiency in both cell lines. These results also suggest that the $[Ca^{2+}]_i$ in 3PC cells is optimal for a high GJIC level, whereas in CA3/7 cells a higher $[Ca^{2+}]_i$ is needed to induce a high GJIC level. The effect of 1.20 mM Ca^{2+}_e on $[Ca^{2+}]_i$ in both cell lines is the same as reported by others for neoplastic keratinocytes (28,29). Kruszewski et al. found that $[Ca^{2+}]_i$ in normal kerationocytes remained at a three times higher level after 22 hours of high $[Ca^{2+}]_e$ compared to the level at low Ca^{2+}_e conditions (29). The $[Ca^{2+}]_i$ of the 3PC cell line however returned also to the basal level. This apparent difference may be a result of the selection procedure of this cell line, i.e. treatment with a initiating agents followed by selection under high extracellular calcium conditions.

Both the calcium ionophore ionomycin and the Ca^{2+}-ATPase inhibitor thapsigargin increased GJIC between CA3/7 cells. This ionomycin-induced rise in GJIC

level was comparable with the high Ca^{2+}_e-initiated GJIC rise after 90 minutes. Therefore, the effect of high Ca^{2+}_e on GJIC in CA3/7 cells could be the result of an increased $[Ca^{2+}]_i$. The GJIC level in 3PC cells remained unchanged after exposure of the cells to ionomycin or Tg. These results suggest that changes can exist in calcium regulated GJIC in cells representing different stages in the process of tumor formation. The kinetics of the ionomycin-induced raise of $[Ca^{2+}]_i$ in 3PC cells is comparable to the high Ca^{2+}_e-induced $[Ca^{2+}]_i$ rise in these cells. In CA3/7 cells however, ionomycin did not induced a first trancient $[Ca^{2+}]_i$ increase. Contrary to these results, Crow et al. (1994) found a decreased GJIC between bovine lens cell cultures when Ca^{2+}_i was increased by ionophores under high Ca^{2+}_e conditions (30), and the calcium ionophore A23187 caused an inhibition of GJIC in **Chironomus** salivary gland cells under high Ca^{2+}_e conditions (31). Furthermore an increased $[Ca^{2+}]_i$ reduced GJIC in hepatoma cells cultured under high Ca^{2+}_e conditions (32). These apparent discrepancies could be the result of the type of cells used.

Because many calcium dependent processes are regulated via the calcium binding molecule calmodulin (CaM), and CaM plays a role in the regulation of GJIC possibly by binding to connexins (15,16) or by activation of connexin-phosphorylation protein kinases (Fig.1), the effect of CaM inhibitors on GJIC was studied in both cell lines. Interestingly, the two CaM inhibitors had opposite effects in 3PC cells and CA3/7 cells. Whereas under low Ca^{2+}_e conditions GJIC was decreased in 3PC cells, GJIC was increased in CA3/7 cells by CaM inhibition. Under high Ca^{2+}_e conditions the GJIC level was not changed by CaM inhibitors in both cell types. Exposure under high Ca^{2+}_e conditions to several CaM inhibitors did not decreased GJIC in different cell types (33-35). This is concistent with our results with both cell lines. However, in Beetle epidermis (36) the GJIC level was decreased by exposure of the cells to CaM inhibitors under high Ca^{2+}_e conditions. The reason for this discrepancy is unknown. The results under low Ca^{2+}_e conditions suggest that changes in CaM-dependent regulation of GJIC may be associated with the process of tumor formation. Because the CaM-induced effect on GJIC is different in both cell lines under low Ca^{2+}_e conditions, this suggests that direct binding of CaM to Cx43 alone is not enough to be a mechanism by which CaM regulates the GJIC level.

Exposure of both cell lines to CDZ or W-7 led to a (transient) $[Ca^{2+}]_i$ increase (Fig 4B). A transient increased $[Ca^{2+}]_i$ was also reported for MDCK cells exposed to the CaM inhibitor trifluoperazine (37). This may be due to inhibition of the CaM-dependent plasma membrane calcium pump (25, 38). In this study we showed that in

3PC cells the $[Ca^{2+}]_i$ was sufficiently high to preserve a high GJIC level, and that an increased $[Ca^{2+}]_i$ did not affect this GJIC level. The results obtained with CaM inhibitors in both cell lines suggest that the effects of CaM on GJIC are more important than the stimulating effects on $[Ca^{2+}]_i$.

Because CaM may exert its effect on GJIC by activation of Ca^{++}/CaM-dependent protein kinase (Ca^{2+}/CaM-PK), and because Saez et al.(1990) showed that activated Ca^{2+}/CaM-PK resulted in an increased Cx32 phosphorylation, we studied the effects of an inhibitor of Ca^{2+}/CaM-PK (ML-7) on GJIC in both cell lines. GJIC was decreased in both cell lines by treatment of the cells with ML-7 under low and high $Ca^{2+}{}_e$ conditions. This suggests that the differences found between 3PC cells and CA3/7 cells in the regulation of GJIC are not due to differences in the regulation of GJIC by Ca^{2+}/CaM-PK. In concurrence with the ML-7-induced GJIC inhibition Cx43 was hyperphosphorylated in both cell lines under both $Ca^{2+}{}_e$ conditions, suggesting no direct phosphorylation of Cx43 by Ca^{2+}/CaM-PK. In addition to the GJIC inhibition by ML-7, $[Ca^{2+}]_i$ was increased. How ML-7 may affect the $[Ca^{2+}]_i$ is however unknown.

$Ca^{2+}{}_i$- or CaM-modulating agents could affect GJIC by activation of Ca^{2+}/CaM-dependent protein kinases and subsequent (in)direct connexin phosphorylation (Fig.1). Here we show that exposure of both cell types to ionomycin or EGTA-AM results in an increased Cx43 phosphorylation. Crow et al.(1994) showed a decreased GJIC in concurrence with a decreased amount of phosphorylated Cx43 in bovine lens cells exposed to calcium ionophores (30). However, in accordance with this study, more phosphate groups per Cx43 were found. Experiments with CDZ and ML-7 showed that CaM-dependent enzymes are involved in the phosphorylation level of Cx43 (Fig 6). However, no clear relationship can be found between Cx43-phosphorylation and effects on GJIC (Table 1). Analysis of the specific amino acid residues of Cx43 phosphorylated by exposure of the cells to Ca^{2+}- or CaM-modulating agents might give more information about the question whether such relationship exists.

The intensity of Cx43-linked immunostaining on the cell membrane was decreased by EGTA-AM, CDZ or ML-7. All treatments of both cell types with modulators of the suppost calcium signal transduction pathway which inhibited GJIC, at the same time affected Cx43 immunostaining levels on the membrane. Because the amount of Cx43 remained unchanged (as determined by Western analysis), this suggests that the used agents cause a dispersion of Cx43 over the membrane, after which the immunofluorescence intensity decreases below detection limits.

The cell adhesion molecule E-cadherin was shown to regulate GJIC in mouse

keratinocytes (14). The functioning of E-cadherin is regulated by adhesion of catenins to E-cadherin (39,40), and appears to be a calcium and phosphorylation related process (41,42). To determine whether E-cadherin regulation is involved in the differences between 3PC cells and CA3/7 cells in the regulation of GJIC, we studied the effects of ionomycin, CZD, and ML-7 on the amount and location (Fig. 7) of E-cadherin protein in both cell lines. None of the tested modulators of the signal transduction route had any effect on the E-cadherin-linked immunostaining on the membranes of the cells, suggesting that the location of E-cadherin is not directly regulated by Ca^{2+}_i-dependent enzymes. The fact that an increased $[Ca^{2+}]_e$ increased the intensity of the E-cadherin immunostaining on the membrane in both cell types, while the amount of E-cadherin remained unchanged, suggests that the E-cadherin molecule underwent a conformational change, or that it became more clustered.

This study shows that differences exist between the 3PC cells and the CA3/7 cells in the regulation of GJIC by Ca^{2+}_e. These differences are not due to differences between the two cell types in the transmembrane transport efficiency of calcium; the regulation of GJIC by Ca^{2+}/CaM-PK, nor by the Ca^{2+}_i/CaM-regulated E-cadherin location. However, differences between the two cell types were found 1) in the requirement for a peak of Ca^{2+}_i to establish full GJIC; and 2) in the CaM-dependent regulation of GJIC. The results of this study suggest that not only the binding of CaM to Cx43 is the mechanism by which CaM regulates GJIC. CaM inhibition has differential effects on the GJIC level in both cell lines. This could be due to the involvement of several different CaM-dependent enzymes in the regulation of GJIC. One possible candidate may be Ca^{2+}/CaM dependent phosphodiesterase, an enzyme which brakes down cAMP (43,44), because of the role of cAMP-stimulated protein kinase A in the regulation of GJIC (45,46).

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Chapter 4

Cyclic amp-mediated control of gap junctional intercellular communication is differentially regulated in initiated and carcinoma cells

This chapter is based on the paper:
L.A.M.Jansen, T.de Vrije, C.van de Berg, and W.M.F.Jongen (1996) Cyclic AMP-mediated control of gap junctional intercellular communication in an initiated- and carcinoma-derived cell line. Submitted to Carcinogenesis.

Abstract

cAMP-mediated regulation of gap junctional intercellular communication (GJIC) was studied in a cell line consisting of initiated cells (3PC) and a carcinoma derived cell line (CA3/7). Under low (0.05 mM) extracellular calcium (Ca^{2+}_e) conditions, forskolin and a specific protein kinase A (PKA) activator increased the low GJIC level of CA3/7 cells, but not the high GJIC level of 3PC cells. Furthermore GJIC was reduced by PKA inhibition in 3PC cells under low, but not under high Ca^{2+}_e conditions. Both PKA inhibition and PKA stimulation could not change the GJIC level in CA3/7 cells under high Ca^{2+}_e conditions. Analysis of the level of connexin43 (Cx43) phosphorylation showed that no direct relationship exists between PKA-modulator-induced change in the percentage of phosphorylated Cx43 and the level of GJIC. Exposure of CA3/7 cells under low Ca^{2+}_e conditions to (in)direct PKA activators raised the GJIC level in concurrence with an increased Cx43 immunostaining on the plasma membrane. Under high Ca^{2+}_e conditions, both the high GJIC level and the Cx43 immunostaining remained unchanged. The amount and localization of the cell adhesion molecule E-cadherin were not changed by treatment of the cells with cAMP/PKA modulators. Finally, no relationship between the cAMP/PKA-induced change in intracellular calcium concentration, and the effect on GJIC was observed. These results demonstrate that differences exist in the cAMP-mediated regulation of GJIC between a cell line consisting of initiated cells and a carcinoma derived cell line.

Introduction

It has been hypothesized that in the multistep process of tumor formation, a decreased gap junctional intercellular communication between genetically altered (initiated) cells and surrounding normal cells can induce clonal expansion (1,2). This may be due to a reduced growth control via gap junctions of adjacent (non-transformed) cells (3,4). Gap junctions are intercellular channels, permitting small metabolites, ions and second messengers to pass to neighbouring cells. How the functioning of gap junctions is regulated is still largely unknown but several groups have suggested that a changed phosphorylation level of the gap junction proteins (connexins) plays an essential role in the inhibition (5,6) or stimulation of GJIC (7). These changes in phosphorylation level can be achieved by (de)activation of protein kinases, however the mechanisms controlling this way of regulation are unknown. Changes in second messenger concentrations like intracellular calcium (8) and

cAMP (9) could be involved. Alterations in these parameters or the responses of enzymes to second messengers could play a part in the presumed role of GJIC in the process of tumor formation. This is supported by the finding of reduced intracellular cAMP concentrations in mouse hepatocytes treated with GJIC-inhibiting agents (10), and by the observation that cAMP stimulating agents can counteract tumor promoter-mediated inhibition of GJIC (10,11). This suggests a role of cAMP or cAMP-dependent enzymes (such as protein kinase A) in the regulation of GJIC. Furthermore, changes in the level of expression and/or the functioning of a cell adhesion molecule, like E-cadherin, could be involved (12).

In mouse keratinocytes during the process of tumor formation, GJIC declines with the progressing stage of this process (12). Elevated extracellular calcium concentrations were capable of restoring GJIC of papilloma and carcinoma derived cell lines to the higher GJIC levels of primary keratinocytes and initiated cells. These differences in Ca^{2+}_e-dependency are partially the result of changed Ca^{2+}_i-dependent processes (Jansen et al., unplubished results). To determine if differences exist between a cell line consisting of initiated cells (3PC) and a carcinoma derived cell line (CA3/7) in the role of cAMP-mediated processes in the regulation of GJIC, we studied the role of cAMP and protein kinase A (PKA) in the regulation of GJIC in these cell lines. In addition we studied the effects of cAMP/PKA-modulating agents on connexin43 phosphorylation and connexin43 localization. Because cAMP/PKA might exert its effect by changing the intracellular calcium ($[Ca^{2+}]_i$) concentration (13-15) we also studied effects of cAMP/PKA-modulating agents on $[Ca^{2+}]_i$ in both cell lines. Finally, the role of cAMP/PKA in the regulation of the amount and localization of the cell adhesion molecule E-cadherin were studied.

Results

Agents which raised the intracellular cAMP level by stimulation of cAMP synthesis (forskolin) or by inhibition of cAMP break down (IBMX) had no effect on the GJIC level of 3PC cells (data not shown) while they increased the GJIC level in the CA3/7 cell line under low Ca^{2+}_e conditions (Fig.1A). Forskolin (50 μM), which raised the GJIC level in CA3/7 cells more effectively within 90 minutes than IBMX (100 μM), did not increase the GJIC level of CA3/7 cells under high Ca^{2+}_e conditions. Because cAMP exerts its effect by activation of protein kinase A (PKA), the effects of a specific activator (SpcAMP) (20), and a specific PKA inhibitor (RpcAMP) (20) on GJIC were studied in both cell lines. Exposure to 100 μM

SpcAMP for 90 minutes, did not change the GJIC level in 3PC cells under low Ca^{2+}_e conditions. SpcAMP increased the GJIC level in CA3/7 cells under low Ca^{2+}_e conditions comparable to the increase of GJIC in forskolin treated cells (Fig.1B). Under high Ca^{2+}_e conditions, SpcAMP did not increase the GJIC level in both cell lines (data not shown for 3PC cells). Inhibition of cAMP-dependent protein kinase by 100 μM RpcAMP had no significant effect on the GJIC level in both cell lines under high Ca^{2+}_e conditions. Under low Ca^{2+}_e conditions, this agent decreased the GJIC level in 3PC cells, and had no effect on the GJIC level in CA3/7 cells (Fig.1C).

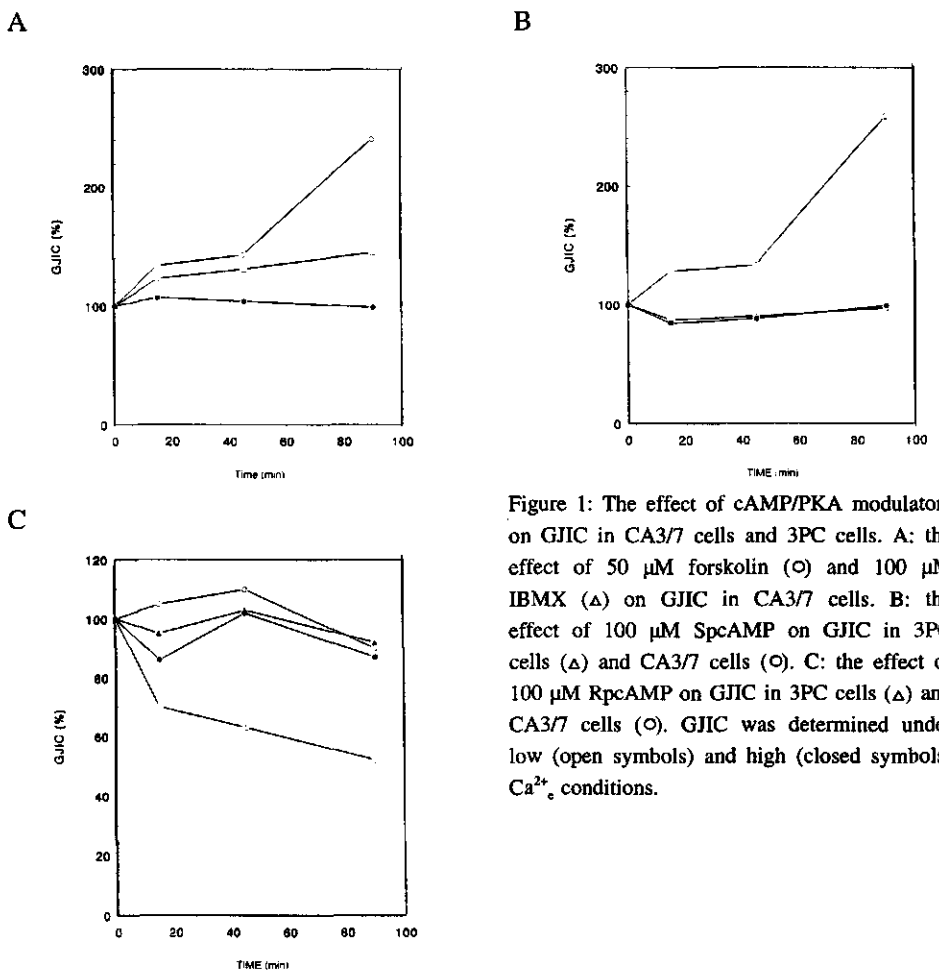

Figure 1: The effect of cAMP/PKA modulators on GJIC in CA3/7 cells and 3PC cells. A: the effect of 50 μM forskolin (○) and 100 μM IBMX (△) on GJIC in CA3/7 cells. B: the effect of 100 μM SpcAMP on GJIC in 3PC cells (△) and CA3/7 cells (○). C: the effect of 100 μM RpcAMP on GJIC in 3PC cells (△) and CA3/7 cells (○). GJIC was determined under low (open symbols) and high (closed symbols) Ca^{2+}_e conditions.

Because cAMP or cAMP-dependent protein kinase can influence the intracellular calcium concentration ($[Ca^{2+}]_i$) (13,14), the effects of forskolin, SpcAMP and RpcAMP on $[Ca^{2+}]_i$ were studied in both cell lines under Ca^{2+}_e conditions in which these agents affected GJIC (Fig.2). In 3PC cells only forskolin significantly increased $[Ca^{2+}]_i$ (Fig.2A). In CA3/7 cells under low Ca^{2+}_e conditions, both SpcAMP and RpcAMP increased $[Ca^{2+}]_i$ significantly, and only after longer exposure times forskolin significantly increased $[Ca^{2+}]_i$ at some time-points (Fig.2B). Under high Ca^{2+}_e conditions, these agents did not affect the $[Ca^{2+}]_i$ in CA3/7 cells.

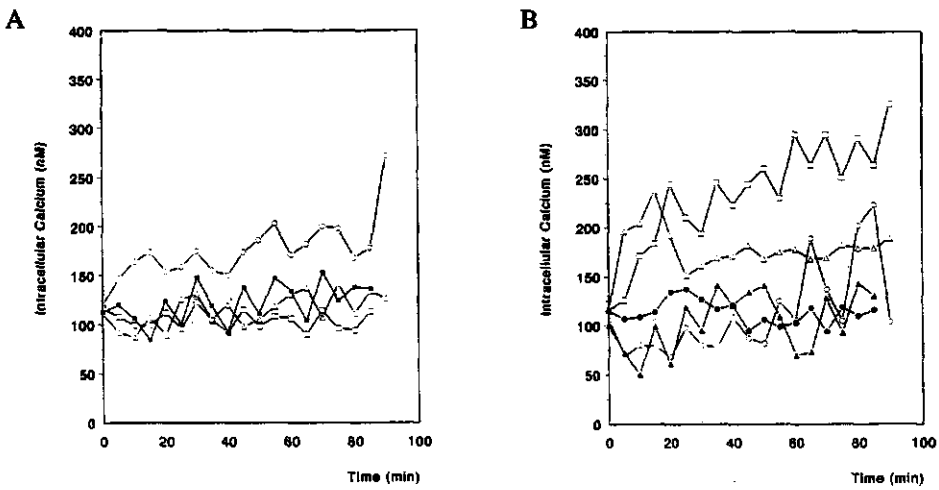

Figure 2: The effect of 50 μM forskolin (○), 100 μM SpcAMP (△), and 100 μM RpcAMP (□) on $[Ca^{2+}]_i$ in 3PC cells (A) and in CA3/7 cells (B) under low (open symbols) and high (closed symbols) extracellular calcium conditions.

PKA could play a role in the phosphorylation of the most abundant gap junction protein in these cells, Cx43 (12). In Fig.3 and Table 1 is shown that forskolin stimulated the phosphorylation of Cx43 in both cell lines under high Ca^{2+}_e conditions, and in 3PC cells under low Ca^{2+}_e conditions. Exposure of CA3/7 cells to forskolin under low Ca^{2+}_e conditions resulted however in a decreased Cx43 phosphorylation. Exposure of 3PC cells to SpcAMP under low Ca^{2+}_e conditions, and CA3/7 cell under both low and high Ca^{2+}_e conditions, resulted in an increased phosphorylation level of Cx43. RpcAMP increased Cx43 phosphorylation in both

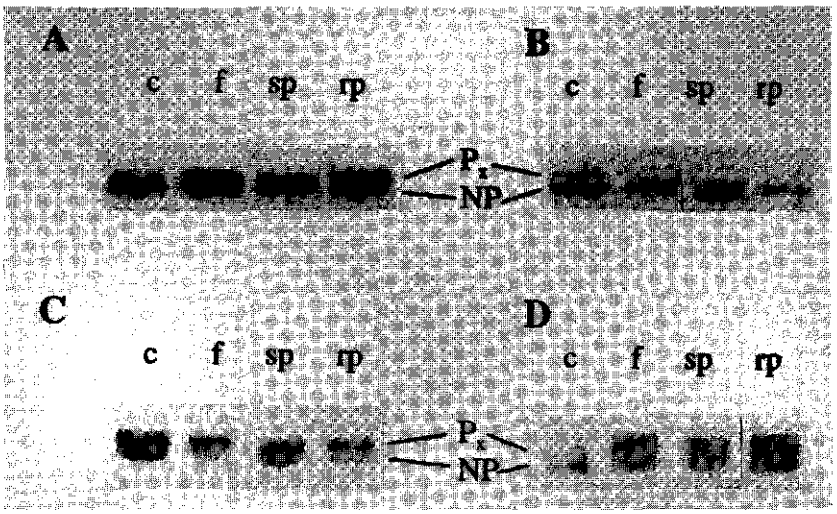

Figure 3: Western blot analysis of connexin43 in 3PC cells (A and C) and in CA3/7 cells (B and D) after 90 minutes of exposure under low (A and B) or high (C and D) extracellular calcium conditions. c=control (untreated) cells, f= 50 μM forskolin; sp= 100 μM SpcAMP; and rp= 100 μM RpcAMP.

	3PC		CA3/7	
Calcium	LOW	HIGH	LOW	HIGH
Control	41	60	28	56
Forskolin (50 μM)	53	77	22	84
SpcAMP (100 μM)	48	66	31	82
RpcAMP (100 μM)	50	70	32	65

Table 1: The effects of cAMP/PKA modulators on the percentage phosphorylated Cx43 in 3PC cells and in CA3/7 cells. The $[Ca^{2+}]_e$ was 0.05 mM and 1.20 mM for low and high calcium medium, respectively.

cell lines under both low and high Ca^{2+}_e conditions. The results of the Cx43 Western analysis are summarised in Table 2.

The location of this gap junction protein might be changed as a result of exposure of the cells to cAMP/PKA-modulating agents. The effects of the used agents on Cx43-linked immunofluorescence under both Ca^{2+}_e conditions are shown in Fig.4 and Table 2. High Ca^{2+}_e conditions increased the intensity of Cx43-linked immunofluorescence in both cell lines on the plasma membrane (Fig.4; A2, B2).

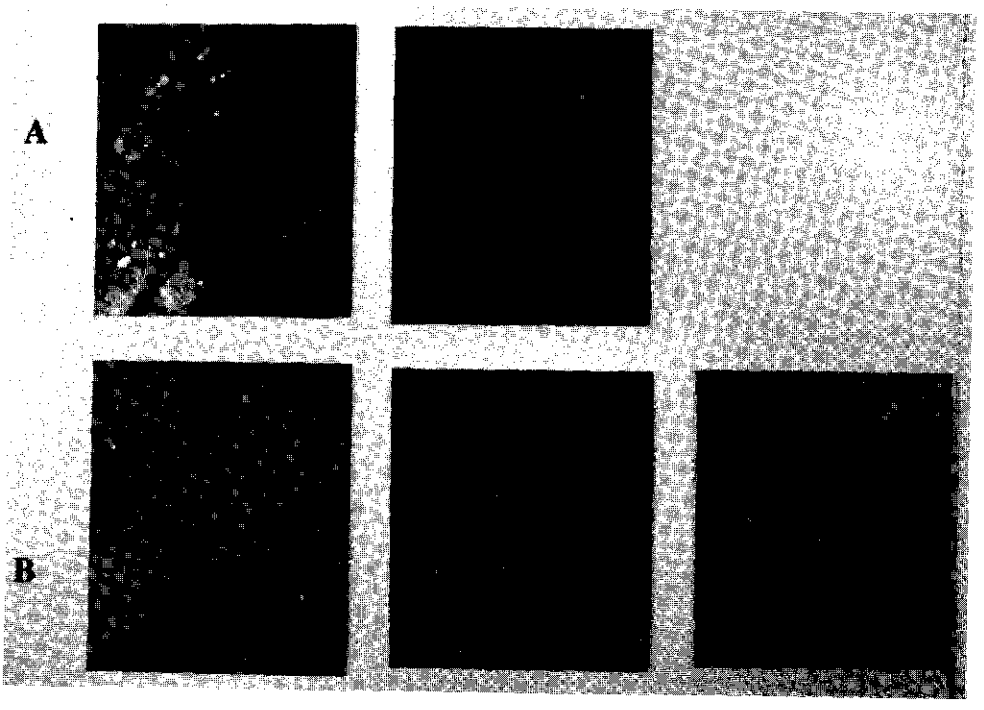

Figure 4: Immunostaining of Cx43 in 3PC cells (A) and in CA3/7 cells (B). Representive pictures are shown of cells under low (A1,B1,B3) or high (A2,B2) Ca^{2+}_e conditions; control staining (A1,B1); a high calcium-induced increased staining (↑ in Table 2, pictures A2,B2); and an increased staining by of forskolin or SpcAMP (↑, picture B3). Narrow arrow heads mark examples of staining on the membranes, broad arrow head marks examples of cytosolic immunostaining. In addition to the specific staining (examples marked by arrow heads), non-specific autofluorescence of the cells, due to the long exposure time of the films, can be seen. Bar = 25 μm.

Forskolin and SpcAMP did not change Cx43-linked immunostaining on the plasma membrane of 3PC cells under both Ca^{2+}_e conditions (data not shown). Both forskolin and SpcAMP increased the intensity of the immunostaining on the plasma membrane of CA3/7 cells under low Ca^{2+}_e conditions (Fig.4; B3), whereas these agents had no effect on the staining intensity under high Ca^{2+}_e conditions (data not shown). Under both Ca^{2+}_e conditions, RpcAMP had no effect on Cx43-linked immunostaining in both cell lines (data not shown).

		3PC			CA3/7		
AGENT (μM)	Calcium (mM)	GJIC[1]	Cx43[2] phosphorylation	Cx43 location[3]	GJIC[1]	Cx43[2] phosphorylation	Cx43 location[3]
Control	0.05	100%	control	++	100%	control	++
Control	1.20	=	control	+++	↑↑	control	+++
Forskolin (50)	0.05	=	↑	=	↑	↓	↑
Forskolin (50)	1.20	=	↑	=	=	↑	=
SpcAMP (100)	0.05	=	↑	=	↑	↑	↑
SpcAMP (100)	1.20	=	=	=	=	↑	=
RpcAMP (100)	0.05	↓	↑	=	=	↑	=
RpcAMP (100)	1.20	=	↑	=	=	↑	=

Table 2: Effect of several agents on GJIC, Cx43-phosphorylation and on Cx43-immunstaining in the cell lines 3PC and CA3/7. 1: Compared to control-L (= 100%) Symbols =,↑,↓ represent an unchanged, an increased or a decreased GJIC respectively; 2: Compared to the control under the same Ca^{2+} conditions, Symbols =,↑,↓ represent an unchanged, an increased or a decreased Cx43-phosphorylation respectively; 3: Compared to the control under the same Ca^{2+} conditions, Symbols =, ↑ represent an unchanged or an increased intensity of immunostaining respectively.

Because in mouse keratinocytes GJIC is also regulated by the cell adhesion molecule E-cadherin (12), we studied the effect of the used cAMP/PKA-modulators on the amount and location of E-cadherin in both cell lines. High Ca^{2+}_e conditions increased E-cadherin immunostaining on the plasma membrane of both cell lines compared to low Ca^{2+}_e conditions, whereas the used cAMP/PKA-modulators had no effect on the immunostaining under both Ca^{2+}_e conditions (data not shown). The amount of E-cadherin was not changed by an increased $[Ca^{2+}]_e$, nor by the used cAMP/PKA-modulators as determined by Western analysis (data not shown).

Discussion

This study shows that differences exist between a cell line consisting of initiated cells and a carcinoma derived cell line in the regulation of GJIC by cAMP-dependent processes. We also demonstrated that these differences can be associated with differences in the cAMP-dependent regulation of the connexin43 localization in the cells. No clear relationship was observed between the level of GJIC and the level of Cx43 phosphorylation. Finally, the amount and localization of the cell adhesion molecule E-cadherin were not directly regulated by modulators of the cAMP/PKA-dependent signal transduction route.

To study possible changes in the cAMP-regulated GJIC in 3PC cells and CA3/7 cells, we exposed both cell types to agents which increase the cAMP concentration and determined the effects on GJIC. The high GJIC level in 3PC cells under both Ca^{2+}_e conditions could not be increased by IBMX and forskolin, whereas the GJIC between the cells with a low GJIC level (CA3/7) could be increased by these agents. The specific activator of PKA, SpcAMP, induced a GJIC increase comparable to the forskolin-induced GJIC increase, suggesting that the forskolin-mediated increase of GJIC is a result of the activation of PKA. Exposure of 3PC cells to the specific PKA inhibitor RPcAMP, resulted in a decreased GJIC level under low Ca^{2+}_e conditions, while under high Ca^{2+}_e conditions the GJIC level remained unchanged. These results suggest that PKA stimulates GJIC. Furthermore, they suggest that a change to high Ca^{2+}_e conditions antagonize against inhibition of GJIC by PKA activity inhibitors. These results support the findings of several groups, that in mammalian cardiac muscle cells and in mouse hepatocytes, the high GJIC level could not be increased by cAMP increasing agents (10,21). Probably, in cells with a high GJIC level, the cAMP-dependent signal transduction route is already optimal stimulated. An increased GJIC after exposure of cells (with a high

GJIC level) to cAMP increasing agents has however been reported for Syrian hamster embryo cells (11,22), and for rabbit gastric epithelial cells (23), suggesting that differences in the role of cAMP/PKA in the regulation of GJIC is cell type dependent.

Because cAMP/PKA can change $[Ca^{2+}]_i$ by either affecting Ca^{2+} release from intracellular stores (13,15) or by effecting membrane Ca^{2+} pumps (24), we determined the effects of cAMP/PKA-modulating agents on $[Ca^{2+}]_i$ in both cell lines. Forskolin increased directly (3PC) or after longer exposure times (CA3/7, after 60-80 minutes) $[Ca^{2+}]_i$ in both cell lines under low Ca^{2+}_e conditions. Under these conditions, the GJIC level remained unchanged (3PC) or was already after 15 minutes of exposure 35% increased (CA3/7). Furthermore both RpcAMP and SpcAMP increased $[Ca^{2+}]_i$ rapidly in CA3/7 cells under low Ca^{2+}_e conditions. The GJIC level in CA3/7 cells was increased by SpcAMP exposure, but not by RpcAMP exposure under these conditions. Furthermore, RpcAMP decreased the GJIC level in 3PC cells under low Ca^{2+}_e conditions, while no effect on $[Ca^{2+}]_i$ was observed. All these results suggests that the effect of cAMP/PKA modulating agents on GJIC is not mediated via changes of $[Ca^{2+}]_i$.

One possible way by which PKA can stimulate GJIC in mouse keratinocytes, is by changing the phosphorylation level of Cx43. The capacity of PKA to phosphorylate gap junction proteins has been reported for Cx32 and Cx26 in hepatocytes (25,26). Activation of PKA in 3PC cells under low Ca^{2+}_e conditions by forskolin or SpcAMP resulted in an increased Cx43 phosphorylation. In CA3/7 cells Cx43 phosphorylation was induced by SpcAMP exposure and not by forskolin exposure. The reason for this discrepancy is unknown. When the effects of the used cAMP/PKA modulators on GJIC are compared with the effects on Cx43 phosphorylation, no association can be found. This is concistent with earlier findings which showed that no relationship exists between the effects of (possible) tumor promoters on GJIC and on Cx43 phosphorylation in the same cell lines (27). Analysis of the phosphorylation on specific amino acid residues of Cx43 after exposure of the cells to cAMP/PKA-modulating agents could give a definite clue to whether such relationship exists at all. The hyperphosphorylation of Cx43 in cells exposed to the PKA inhibitor RpcAMP suggests that Cx43 is phosphorylated by (an) other protein kinase(s), whose activity is (in)directly regulated by PKA.

Besides effects on Cx43 phosphorylation, PKA might change GJIC by changing the location of Cx43 in the cells. Forskolin and SpcAMP increased the

intensity of Cx43 immunostaining in CA3/7 cells under low Ca^{2+}_e conditions, which correlates with an increased GJIC level. This is consistent with the findings that the Cx43-linked immunostaining on membranes of hepatocytes could be increased by forskolin (28) and by 8-BrcAMP treatment (29). No forskolin- or SpcAMP-induced increase of immunofluorescence intensity could be observed in 3PC cells under low Ca^{2+}_e conditions, nor in both cell lines under high Ca^{2+}_e conditions, which correlates with the observed effects on GJIC. These results confirm the findings of Mehta et al. (28) that in hepatocytes with a basal high GJIC level, both GJIC and the amount of Cx43 mRNA could not be increased by forskolin treatment.

In conclusion this study shows that differences exist between a cell line consisting of initiated cells and a carcinoma-derived cell line in the regulation of GJIC by cAMP/PKA-modulating agents. Under conditions in which the GJIC level was low in CA3/7 cells, GJIC could be increased by PKA-activating agents, in concurrence with an increased Cx43 staining on the cell membrane. A high GJIC level in both cell types could not be further increased by PKA-activating agents. Under low Ca^{2+}_e conditions, GJIC could be inhibited in 3PC cells by PKA-inhibition. Finally, no relationship was found between effects of cAMP/PKA-modulating agents on GJIC and their effects on $[Ca^{2+}]_i$ or Cx43 phosphorylation.

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Chapter 5

The use of initiated cells as a test system for the detection of inhibitors of gap junctional intercellular communication

This chapter is based on the paper: L.A.M.Jansen and W.M.F Jongen (1996) The use of initiated cells as a test system for the detection of inhibitors of gap junctional intercellular communication. Carcinogenesis, 17, 333-339.

Abstract

The effects of five non-mutagenic carcinogens 1) Aroclor 1260, 2) benzoyl peroxide (BP), 3) phenobarbital (PB), 4) 12-o-tetradecanoyl-phorbol-13-acetate (TPA) and 5) 1,1'-(2,2,2-trichloroethylidene)bis[4-chlorobenzene] (DDT) on gap junctional intercellular communication (GJIC) were tested in a cell line consisting of initiated cells (3PC). Four agents suspected of tumor promotion activity 1) o-anisidine, 2) clofibrate, 3) L-ethionone and 4) d-limonene were also tested for their effects on GJIC. Finally sodium fluoride (NaF), of which the carcinogenic property is still unclear, was tested for its effects on GJIC in the 3PC cell line. Four from the five selected tumor promoters (Aroclor 1260, BP, DDT and TPA) decreased GJIC between these initiated epidermal cells. The four non-mutagenic carcinogens with tumor promoting activity in vivo (o-anisidine, clofibrate, L-ethionine and d-limonene) all inhibited GJIC, whereas NaF had no effect. Seven compounds (o-anisidine, aroclor 1260, BP, DDT, L-ethionone, d-limonene and TPA) had a dose-dependent as well as time-dependent inhibitory effect on GJIC. Under the experimental conditions used, clofibrate showed only a dose-related inhibition of GJIC. PB showed no inhibitory effect on GJIC in the 3PC cell line. In order to determine the role of biotransformation in the tumor promoting activity of PB, its effect on GJIC was also examined in the presence of an aroclor 1254 induced rat liver homogenate (S9 mix) and in the hepatoma cell line HepG2. In the presence of rat liver homogenate PB decreased GJIC in the 3PC cell line, whereas in the HepG2 cells PB showed a time- and dose-dependent inhibitory effect. To study the potential differences in susceptibility of cells representing different stages in the process of tumor formation the effect of the selected tumor promoters on GJIC were also investigated in primary mouse keratinocytes and in a mouse skin carcinoma derived cell line (CA3/7). Primary keratinocytes were sometimes more (BP and clofibrate) and sometimes less sensitive (ethionin and limonene) for inhibitory effects on GJIC compared to the effects in the cell line 3PC. Except for TPA and anisidin, GJIC between the CA3/7 cells was less affected by the selected agents compared to the 3PC cell line. Firstly, these results show that, during the process of tumor formation the susceptibility of cells to inhibition of GJIC by tumor promoters is variable. Overall the CA3/7 cells are less sensitive compared to 3PC cells. The susceptibility of primary keratinocytes is variable compared to 3PC cells, depending on the agent used. Secondly, these results show that GJIC is a valid parameter for testing tumor promotion activity of compounds. And finally, this study demonstrates, that mouse

keratinocyte cell lines could serve as an in vitro model for the detection of non-mutagenic carcinogens with diverse target organs in vivo. For this use the cell line consisting of initiated cells (3PC) is more sensitive than the carcinoma derived cell line CA3/7.

Introduction

Chemical induced tumor formation is a process in which various stages can be distinguished. During the initiation stage, a carcinogen-induced mutation of the DNA takes place. The following promotion stage involves processes in which initiated cells undergo clonal expansion to form premalignant lesions. It has been postulated that inhibition of intercellular communication may be a causal factor in this process. Several lines of evidence suggest an important role for gap junctional intercellular communication (GJIC) in the process of tumor formation. Firstly, a large proportion of compounds with tumor promoting capacity were shown to decrease GJIC in various mammalian cell types (1-3). Secondly, several groups (4-7) reported a lack of GJIC between transformed and non-transformed cells permitting transformed cells to maintain autonomous growth. In the third place, by stimulation of junctional communication with normal cells, the transformed phenotype of transformed cells can be suppressed (5,8).

One important aspect of tumor promoters is their tissue specificity. The toxico-kinetic behaviour as well as the metabolism of compounds in the target organ are important determinants of the toxicity (or carcinogenicity). Therefore, the use of cells from the target tissue forms the basis of several in vitro systems to determine the effect of compounds on GJIC. Some of these systems use primary cell cultures (9-11) whereas others use cell lines like the Chinese hamster V79 cell line (1). Our working hypothesis is that the effect of agents, suspected of tumor promoting activity on GJIC, should be tested in a system consisting of cells which are the target cells for tumor promoters, i.e. initiated cells. These cells could be more sensitive to the effects of tumor promoters (12,13). To test this hypothesis we used the mouse epidermal cell line 3PC which was derived from primary mouse keratinocytes, exposed to DMBA in vitro and selected under high calcium conditions (14). These cells are considered initiated cells because of their resistance to the differentiation-inducing effect of calcium, which has been suggested to be a critical event associated with initiation of carcinogenesis in mouse skin (15), and on the other hand have a GJIC level at low calcium conditions comparable with primary

keratinocytes (16). Because biotransformation activity of 3PC cells is very low, compounds not directly affecting GJIC in the 3PC cell line were tested for their potential to inhibit GJIC in two ways: A) in the presence of an aroclor 1254 induced rat liver homogenate, and B) in the hepatoma cell line HepG2 (17). In this way, both the direct effect on GJIC and the effects after biotransformation of compounds can be determined. To validate our system we tested five non-genotoxic carcinogens with known capacity to decrease GJIC in in vitro systems (Table 1). Furthermore, four non-genotoxic agents with carcinogenic capacity in vivo, and one agent of which the carcinogenic property is still unclear (NaF), were tested for their effect on GJIC in the mouse epidermal cell line 3PC.

Table 1: Literature overview of tumor related properties of the selected agents.

Agent	Inhibition GJIC in vitro[a]	Mutagenicity (S. typhimurium assay-test)[b]	Tumor promoter/ carcinogen in vivo[d]	Main target tissue in vivo
Aroclor 1260	+ 18,19	+/- 20 - 21-23	+ 21,24	liver
BP	+ 1,25 - 26	- 23,26,28	- 28 + 25,29,30	skin
DDT	+ 2,18,19,31	- 19,32	+ 33-35	liver
TPA	+ 2,18,19	- 36,37	+ 30,38,39	skin
PB	- 2,40,41 + 1,18,32,42	- 43	+ 33,44-45	liver
o-Anisidine	?	+ 46,47[c] - 48	+ 47,49	bladder
L-Ethionine	?	- 50	+ 50,51	liver
d-Limonene	?	- 43	- 52-54 + 55,56	kidney
Clofibrate	?	- 57	+ 58-60	liver
NaF	?	- 61	- 62 + 63	bone

a) + = ↓ GJIC, - = ↑ or no effect on GJIC, ? = unknown.

b) - = not mutagenic; +/- = only weakly mutagenic.

c) only mutagenic after metabolic activation (46,47).

d) + = tumor promotion or carcinogenic action, - = no such action.

Table 2: Inhibition of GJIC in the cell line 3PC after exposure to several compounds.

Agent	Concentration (µg/ml)	GJIC (percentage of control) Time (min)									
		7.5		15		45		70		90	
Aroclor 1260	200			91		35	*	10	*	5	*
	500			82		55	*	8	*	13	*
	1000			28	*	10	*	6	*	tox	
Benzoyl Peroxide	10.0			107		74	$	47	*	35	*
	40.0			82		59	*	37	*	29	*
	80.0			71	*	53	*	28	*	23	*
DDT	2.0			99		78		55	*	55	*
	5.0			84		62	*	52	*	42	*
	10.0			83		27	*	24	*	7	*
TPA	1.0			57	*	49	*	66	*	66	*
	2.0			79	$	62	*	46	*	47	*
	4.0			69	*	27	*	20	*	22	*
PB	500			90		90					
	1000			92		101				105	
o-Anisidine	1232	111		79		76		79		70	$
	1848	76	*	78	*	59	*	37	*	38	*
	2464	43	*	75	*	27	*	23	*	21	*
L-Ethionine	204	88		99		50	*	64	$	58	*
	408	69	*	40	*	41	*	48	*	47	*
	816	52	*	40	*	35	*	33	*	28	*
d-Limonene	136	22	*	33	*	27	*	22	*		
	272	13	*	9	*	10	*	6	*		
	681	6	*	2	*	3	*	tox			
Clofibrate	51	23	*	32	*	22	*	15	*		
	126	10	*	8	*	6	*				
	250	5	*	9	*	6	*				
NaF	420			112		113					
	1050			96		96					
	2100			101		83		97			

Significantly different from control (100%) bij Analysis of Variance (ANOVA) (* = $p \leq 0.001$, $ = $p \leq 0.01$)

To study the changes in susceptibility during the process of tumor formation for inhibition of GJIC by tumor promoters, we compared the effects of these non-genotoxic carcinogens on GJIC in 3PC cells with those in primary mouse skin keratinocytes and a mouse skin carcinoma derived cell line (CA3/7).

Results

The agents used in this study are considered to be non-mutagenic carcinogens due to their non-mutagenicity in S. typhimurium and their carcinogenic effects in in vivo experiments (summarized in Table 1). Table 2 shows that four of the five compounds known to decrease GJIC in various test systems had also inhibitory effects on GJIC in the mouse epidermal cell line 3PC (Aroclor 1260, BP, DDT, TPA). The four compounds selected for their non-mutagenic carcinogenic effects in vivo (o-anisidine, clofibrate, L-ethionone and d-limonene) all decreased GJIC in the 3PC cell line. Only the tumor promoter phenobarbital and NaF did not affect GJIC. From the agents with inhibitory effects on GJIC, seven compounds showed a time- as well as dose-dependent inhibition of GJIC (Table 2). Under the experimental conditions used here, clofibrate showed only a dose-dependent inhibition of GJIC. At all concentrations tested, both PB and NaF did not significantly decrease GJIC after 90 minutes (Table 2).

Cell line	Concentration PB (µg/ml)	S9	GJIC (% of control)			
			Time (min.)			
			15	#	45	#
3PC	0	+	94		97	
3PC	1000	-	100		107	
3PC	1000	+	63	***	58	***
HepG2	500	-	68	*	42	***
HepG2	1000	-	40	***	nd	

Table 3: GJIC in the cell lines 3PC and HepG2 after exposure to PB. # Significantly different from control (100%) bij Analysis of Variance (ANOVA) (* = $p \leq 0.03$, *** = $p \leq 0.001$), nd = not determined, loss of cell viability

To determine if PB needs metabolic activation to affect GJIC, the effect of PB on 3PC cells was tested in the presence of aroclor 1254 induced rat liver

homogenate (S9 mix). Addition of S9 mix did inhibit GJIC during exposure of 3PC cells to PB (Table 3). The effect of PB on GJIC was also determined in the hepatoma cell line HepG2. These cells were shown to have cytochrome P450 enzyme activity (17) whereas the metabolic capacity of the 3PC cells is very low (data not shown). In the cell line HepG2, PB and/or PB metabolites inhibited GJIC in a dose- as well as time-dependent manner.

To study the involvement of extracellular calcium in the inhibition of GJIC, 3PC cells were cultured for 24 hours in high calcium medium (1.2 mM). As for the low calcium conditions, cells were exposed to tumor promoters and the effect on GJIC was determined. Except for a slight decreased sensitivity for BP induced GJIC inhibition, high extracellular calcium conditions had no effect on the inhibition of GJIC by the tumor promoters (data not shown).

To study potential differences in susceptibility of cells representing different stages in the process of tumor formation, the effect of the selected tumor promoters on GJIC was tested in mouse primary keratinocytes and in a mouse skin carcinoma derived cell line (CA3/7). Table 4 shows that all tumor promoters which inhibited GJIC in the 3PC cell line, also affected GJIC in the carcinoma derived cell line CA3/7. Except for TPA and anisidin, inhibition of GJIC by the tumor promoters was significantly less in the CA3/7 cell line compared to the 3PC cell line, but still in the same order of magnitude. In primary keratinocytes GJIC was significantly more inhibited at short exposure times by BP, clofibrate and DDT, although at 90 minutes of exposure to DDT the degree of GJIC inhibition was comparable with the results in the 3PC cell line. In the primary cells, TPA and Aroclor 1260 caused only at longer exposure times a stronger, respectively less strong inhibition of GJIC compared to the effect obtained with 3PC cells. Limonene had a much weaker effect on GJIC in primary cells compared to the effect in both cell lines. Finally, ethionine did not significantly affect GJIC in primary keratinocytes.

Discussion

In the present study, we used initiated cells to detect non-mutagenic carcinogens in vitro, based on the hypothesis that these cells are the target cells for tumor promoters. Four out of five tumor promoters with known GJIC-inhibiting capacity, decreased GJIC in the cell line consisting of initiated mouse epidermal cells (Table 2). Using higher (non-toxic) concentrations (BP, TPA) or the same concentrations (Aroclor 1260, DDT) compared to others (1,17-19,25,26,31), we could detect

Tabel 5: Remaining percentage (control = 100%) of GJIC in cell treated with different tumor promoters.

AGENT (µg/ml)	TIME (min)	3PC	CA3/7	primimary keratino-cytes
Aroclor 1260 (200)	15	82	79**	76*
	45	55*	56*	46*
	70	8*	37*,***	NT
	90	13*	40*,***	30*,***
Benzoyl Peroxide (40)	15	82	97	15*,***
	45	59*	73*,***	13*,***
	70	37*	54*,***	NT
	90	29*	48*,***	NT
DDT (5)	15	84	90	69**,***
	45	62*	72*	41*,***
	70	52*	66*,***	NT
	90	42*	68*,***	52*
TPA (2)	15	79**	66*	55*
	45	62*	50*	36*,***
	70	46*	31*	NT
	90	47*	37*	25*,***
Phenobarbital (1000)	15	92	107	NT
	45	101	106	104
	90	105	96	98
Anisidine (1848)	15	78*	81**	61*,***
	45	59*	67*	49*
	70	37*	47*,***	NT
	90	38*	47*	45*
Clofibrate (51)	15	30*	61*,***	12*,***
	45	22*	55*,***	5*,***
	70	15*	42*,***	NT
Ethionine (408)	15	40*	62*,***	100***
	45	41*	71*,***	115***
	70	48*	68*,***	NT
	90	47*	69*,***	120***
Limonene (136)	15	33*	53*,***	67**,***
	45	27*	50*,***	81***
	70	22*	43*,***	88***

* = significantly different from control in same cell type, $p \leq 0.001$; ** = significantly different from control in same cell type, $p \leq 0.01$; *** = significantly different from remaining GJIC in 3PC cell line under the same conditions, $p \leq 0.001$.

inhibition of GJIC after shorter exposure times. The fifth positive control, phenobarbital, decreased GJIC only after metabolic activation by aroclor 1254-induced rat liver homogenate. Several groups have reported inhibition of GJIC after exposure of cells to PB (see Table 1) whereas others did not find decreased GJIC in Chinese hamster V79 fibroblasts (2,41). One possible explanation is that PB needs to be metabolized and that the metabolites inhibit GJIC. In liver cells, enzyme systems involved in biotransformation are sufficiently active. Indeed, inhibitors of cytochrome P450 monooxygenase prevented inhibition of intercellular communication by PB (65,66), suggesting a role of PB-metabolites in the PB-induced inhibition of GJIC. As we demonstrated with DDT and Aroclor 1260, the DMBA-initiated mouse epidermal cell line 3PC is sensitive for the inhibitory effect of direct acting liver tumor promoters on GJIC.

To clarify the contradictory findings with PB we first incubated PB with an aroclor 1254-induced rat liver homogenate to convert PB into its metabolic products. Exposure of 3PC cells to these metabolites decreased GJIC to almost 50% of control levels. In HepG2 hepatoma cells (with sufficient metabolic capacity), PB affects GJIC without the presence of S9 mix (Table 3). These results point to an essential role of metabolic activation of PB to affect GJIC. It shows the need for appropriate metabolizing systems in the screening of the potential of agents to inhibit GJIC.
Four compounds with established tumor promoting activity, based on data of in vivo carcinogenicity and non-mutagenicity, were selected and tested for their effect on GJIC in the 3PC cell line. No information is available as to whether the effects of these agents on GJIC in other systems is tested. All four compounds decreased GJIC in a dose-dependent way in the 3PC cell line (Table 2). Except for clofibrate, they also showed a time-dependent inhibition of GJIC in 3PC cells.

3PC cells were shown to be resistant for the induction of differentiation by an increase of extracellular calcium (14). The level of GJIC in these cells is also not influenced by a change of extracellular calcium from 0.05 to 1.2 mM (16). Except for a slight decreased sensitivity for BP induced GJIC inhibition, high extracellular calcium conditions did not affect inhibition of GJIC by the selected tumor promoters compared to low calcium conditions (data not shown). This finding agrees with the results of other groups obtained with TPA and DDT (73,86). Madhukar et al showed however that TPA as well as DDT increased intracellular calcium in hamster fibroblasts (87). This suggests that mobilisation of calcium from intracellular stores may affect GJIC, as the non-phorbol ester tumor promoter thapsigargin does (88,89).

To determine if initiated cells are more susceptible for inhibition of GJIC then cells in other stages of carcinogenesis, we compared the effects of several agents on GJIC in primary keratinocytes and in a cell line of carcinoma cells (CA3/7, Table 4). The results presented here show that the cell line of initiated cells (3PC) is more sensitive for inhibition of GJIC by almost all selected tumor promoters than the carcinoma derived cell line CA3/7. The primary keratinocytes were sometimes more (for BP, clofibrate, and in a lesser extend TPA) or less (aroclor 1260, ethionone and limonene) sensitive for inhibition of GJIC by tumor promoters than the 3PC cells. GJIC in primary cells was at shorter exposure times more affected by DDT then in 3PC cells, but this difference disappeared after 90 minutes of exposure. All together, this suggest that during the process of tumor formation, depending on the tumor promoter, changes in susceptibility for inhibition of GJIC can occur, and that the type of change is strictly confined to the agent used.
Using some of the known tumor promoters, several mechanisms involved in the inhibition of GJIC are studied. Both hyper- and hypophosphorylation of connexins are related with changes in levels of GJIC (ref 67-70). The changed phosphorylation level of connexins may be a result of the (in)activation of protein kinases. The involvement of protein kinases and connexin phosphorylation in the inhibition of GJIC has been reported for TPA (71-75), DDT (71,75-77) and PB (73).
Hyperphosphorylation may affect the functionality of gap junction channels, but it may also play a role in the transport of connexins from and to the cell membrane (78). Changes in connexin localization was reported in cells treated with TPA (69,71,74), PCB (79), PB (79) and DDT (71,75), although other reports showed no changes in connexin localization after treatment of cells with TPA (71,73) or DDT (71,75). These differing findings may result from the different cell types used. An other way tumor promoters may affect GJIC is by the production of reactive oxygen radicals as demonstrated for TPA (81-83), PB (82,84), BoP (25,81) and DDT (82). These products may cause oxidation of membrane lipids, hereby influencing membrane fluidity and subsequent gap junction plaque stability (80). The peroxisome proliferator clofibrate (85) may also affect GJIC by reactive oxygen products produced in the peroxisomes. Because this is the first time that inhibition of GJIC by anisidin, clofibrate, ethionine and limonene is demonstrated, no work is done so far on the mechanisms of inhibition of GJIC by these compounds.

All together, these results show that the DMBA-initiated mouse epidermal cell line 3PC can be used as a short-term model for detecting tumor promoters with

different target organs in vivo. These results also demonstrate that, during the process of tumor formation, changes in susceptibility for inhibition of GJIC by tumor promoters occur. The mechanisms underlying these changes could be an interesting subject for further research. Finally these results show that o-anisidine, L-ethionone, d-limonene and clofibrate, but not NaF are potential tumor promoters.

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Chapter 6

Inhibition of gap junctional intercellular communication and delocalization of the cell adhesion molecule E-cadherin by tumor promoters

This chapter is based on the paper:
L.A.M.Jansen, M.Mesnil, and W.M.F.Jongen (1996) Inhibition of gap junctional communication and delocation of the cell adhesion molecule E-cadherin by tumor pormoters. Carcinogenesis, 17, 1527-1531.

Abstract

The effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and benzoyl peroxide (BoP) on gap junctional intercellular communication (GJIC) and the amount and localization of E-cadherin was studied in initiated mouse epidermal cells (3PC) and in carcinoma cells (CA3/7) originating from the same cell type. In addition, the localization and phosphorylation of connexin43 was studied in both cell lines and in primary keratinocytes. GJIC inhibition by TPA and BoP was stronger in primary keratinocytes compared to both cell lines. BoP strongly decreased the amount of E-cadherin protein and the level occurring in the membranes in both cell lines, whereas TPA caused a translocation of E-cadherin from the membrane towards the cytosol, without decreasing the total amount of E-cadherin present. The effect of both tumor promoters on connexin43 phosphorylation and localization was agent- as well as cell-dependent. These results show for the first time, that tumor promoters can decrease the quantity and membrane localization of E-cadherin in different cell types.

Gap junctions are intercellular membrane channels, permitting the free exchange of small molecules (< 1000 dalton) between the cytosol of neighbouring cells. They are thought to play an important role in the maintenance of cell differentiation and homeostasis (1). In the multistage process of carcinogenesis, inhibition of gap junctional intercellular communication (GJIC *) is considered to be an important event during the promotion stage, since it has been shown to be inhibited by various types of tumor promoters (2). The mechanisms by which these agents decrease GJIC include changes in gap junction protein (connexin) localization and phosphorylation as was shown both in vitro (3) and in vivo (4). The cell line 3PC, consisting of initiated mouse keratinocytes, has been shown to be a useful tool for testing the tumor promoting capacity of agents (5). These cells are more sensitive for the inhibition of GJIC by tumor promoters compared to the mouse skin carcinoma-derived cell line CA3/7. Primary keratinocytes are, depending on the tumor promoter used, either more or less sensitive than 3PC cells. These results suggest changes in susceptibility of cells for the inhibition of GJIC by tumor promoters during the process of tumor formation.

Since in mouse keratinocytes GJIC is also regulated by the cell adhesion molecule E-cadherin (6), and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was shown to inhibit cell assembly in various cell types (7), we studied the effect of two tumor promoters (TPA and benzoyl peroxide (BoP)) on the quantity and localization of E-cadherin in the cell lines 3PC and CA3/7 in relation to effects on

GJIC levels. Mouse primary keratinocytes were harvested and cultured as described (6). The cell lines 3PC and CA3/7 were cultured as previously described (8). To study the mechanisms of TPA- and BoP-mediated inhibition of GJIC, we first determined the effect of both tumor promoters on GJIC levels in the three cell types by the fluorescent dye transfer method (6) (Table 1).

	TIME (min)	GJIC (% inhibition)		
		Prim.Ker.	3PC	CA3/7
TPA (4 µg/ml)	15	45	21	34
	45	64	38	50
	90	75	53	63
BoP (40 µg/ml)	15	85	18	3
	45	87	41	27
	90	nd	71	52

Table 1: Percentage of inhibition of GJIC in different cell types after exposure to TPA or BoP. The number of recipient cells under control conditions (18, 30, and 25 for prim.ker., 3PC and CA3/7 cells, variation < 7%) was not changed by the solvents used. The solvents and tumor promoters used, had no influence on cell viability as determined by the Trypan blue exclusion test. The GJIC capacity is expressed as the percentage GJIC inhibition compared to control GJIC (100% at t=o). Prim.Ker.= primary keratinocytes, nd= not determined.

After 90 minutes of exposure to both tumor promoters, the number of communicating cells was decreased in both cell lines. Primary keratinocytes were much more sensitive for inhibition of GJIC by BoP compared with the two cell lines. For further studies on the effects of TPA (4 µg/ml) and BoP (40 µg/ml) on connexin43 (Cx43) and E-cadherin, cells were exposed for 90 minutes to the agent (except the exposure of primary keratinocytes to BoP (45 minutes)), after which either total protein was extracted for Western analysis, or the cells were used for immunofluorescence experiments. We studied the effects of exposure on the amount of E-cadherin protein as examined by Western analysis (4), using for E-cadherin a 10% polyacrylamide gel and a first antibody from Sigma (St Louis, MO). As shown in Figure 1, TPA had no

effect on the amount of E-cadherin protein in all cell types tested. However, after 90 minutes of exposure to BoP, no E-cadherin protein was detectable in both cell lines, whereas in primary keratinocytes the amount of E-cadherin protein was strongly decreased (after 45 min) compared to control cells. The film of blot A (Fig.1) was exposed longer than those exposed to blot B and C. The differences in band intensity between different cell types therefore suggest a lower amount of E-cadherin protein/mg total protein in 3PC cells compared to the other cell types.

Immunofluorescence techniques were used to study the localization of E-cadherin protein in the cell lines 3PC and CA3/7. The technique used was the same as described for connexin43 (4), using the same anti-E-cadherin antibody as for the Western analysis. In both cell lines, under control conditions, the main proportion of E-cadherin-linked immunofluorescence was found on cell-cell contact areas (Fig 2; A2, B2: narrow arrow heads). TPA caused a strong decrease in immunofluorescence on these contact areas and an increase in immunostaining around the nucleus (3PC cells) or in the cytosol (CA3/7 cells) (Fig 2; A3, B3: broad arrow heads). This suggests an effect of TPA on E-cadherin localization in the cells, irrespective of the cell line used. BoP also caused a strong decrease in membrane immunofluorescence but

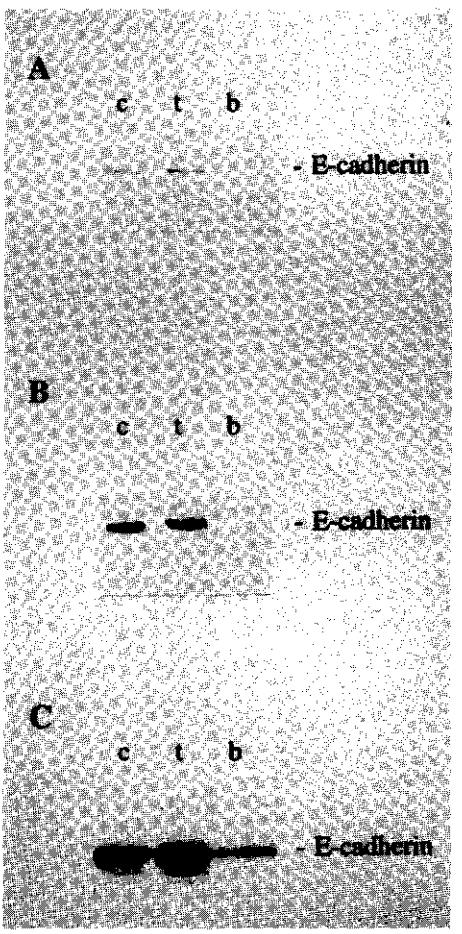

Fig 1. Western blot analysis of E-cadherin in 3PC cells (A), CA3/7 cells (B) and primary keratinocytes (C). c= control (untreated) cells, t= TPA (4 µg/ml) treated cells, b=BoP (40 µg/ml) treated cells. Cells were exposed for 90 min to TPA or BoP, except primary keratinocytes exposed to BoP (45 min). 20 µg protein per sample was applied for each lane.

some (point) staining was present in the cytosol of both cell lines (Fig 2; A4, B4: broad arrow heads). This immunocytochemistry staining is specific because no staining was found in both cell lines when PBS was used instead of a first antibody (Fig. 2; A1, B1). The intensity of E-cadherin immunostaining in BoP-treated cells is however much lower compared to the clear membrane staining in untreated cells of both cell lines, supporting the findings of a strongly decreased amount of E-cadherin as detected by Western blotting analysis. The change in localization of E-cadherin caused by TPA and BoP suggests that these tumor promoters disturb the function of this cell adhesion molecule. This could result in an increase of intercellular space, limiting the

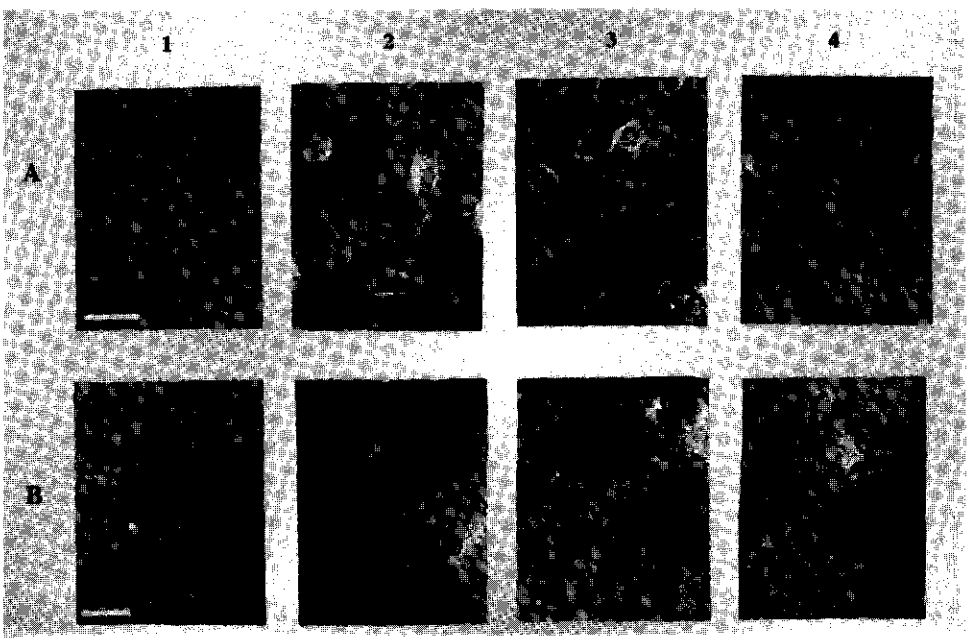

Fig 2. Immunostaining of E-cadherin in 3PC cells (A) and CA3/7 cells (B). Staining was performed with 1) untreated cells without first antibody (negative control), 2) untreated cells with first antibody (positive control), 3) TPA (4 µg/ml, 90 min.) treated cells and 4) BoP (40 µg/ml, 90 min.) treated cells. Bar is 25 µm in both series A and B. The film of picture 1A and 1B was exposed for a longer time, causing a non-specific autofluorescence of the cells in these pictures. Narrow arrow heads mark examples of staining on the membrane between cells, broad arrow heads mark examples of cytosolic staining in cells.

possibility of gap junction channel formation (7). In this study no apparent change in cell assembly was observed, which may be due to the fact that the cells were still well attached to the culture dishes during the exposure to the tumor promoters.

The mechanisms by which tumor promoters affect E-cadherin functioning are still unclear, but (in)activation of E-cadherin bound proteins (like catenins or APC (Adenomatous Polyposis Colon gene)) may play a role. Catenins are proteins which bind to E-cadherin, and which are involved in the functioning of E-cadherin (9-12). The phosphorylation of ß-catenin on tyrosine has been shown to decrease the E-cadherin functioning (13,14). Tumor promoters could change the binding of catenins to E-cadherin by activation of tyrosine kinases or inactivation of tyrosine phosphatases, thereby decreasing cell recognition and gap junction formation. Another molecule binding indirectly to E-cadherin is the tumor suppressor molecule APC. This molecule has been shown to associate with catenins (15,16), suggesting a possible role of APC in the regulation of E-cadherin or vice versa. The effect of tumor promoters on E-cadherin function may generally be caused by affecting APC-like activity, or the decrease of E-cadherin function may affect APC-

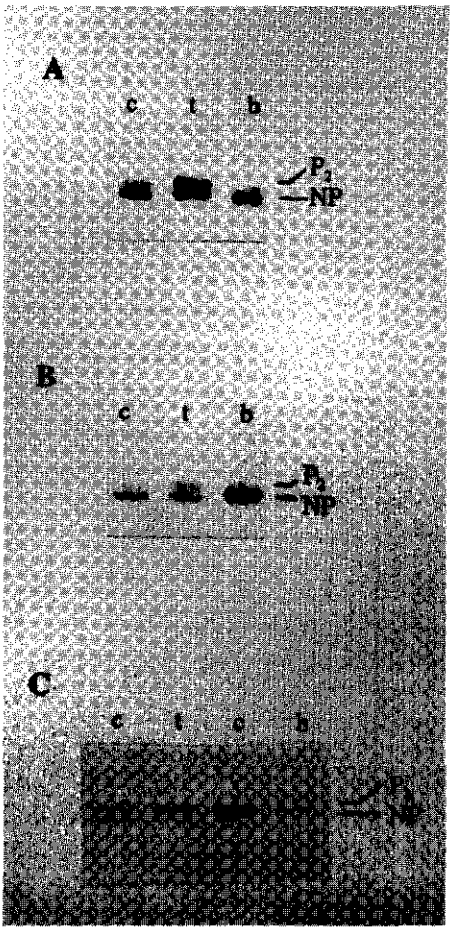

Fig 3. Western blot analysis of connexin43 in 3PC cells (A), CA3/7 cells (B) and primary keratinocytes (C). c= control (untreated) cells, t= TPA treated cells, b=BoP treated cells. NP=non-phosphorylated Cx43, P_x= phosphorylated Cx43. Times of treatment and concentrations were as described in Fig.1. 20 µg protein per sample was applied for each lane.

regulated tumor suppression.

TPA is known to activate protein kinase C (PKC) (17,18), which was shown to decrease the expression of intercellular adhesion molecule 1 (ICAM-1) (19). Lewis et al (20) showed that E-cadherin could organise various junctional components through PKC. Thus, PKC activity could be changed by tumor promoters directly, as well as indirectly by changes in E-cadherin functioning. Furthermore both TPA and BoP increase the amount of reactive oxygen species (for references see L.Jansen and W.Jongen, in press) which have been shown to decrease protein tyrosine kinases and induce translocalization of E-cadherin in MDCK cells (21).

Changes in phosphorylation levels or localization of connexins have been reported to occur in cells exposed to tumor promoters (3,4). Therefore, the effect of TPA and BoP on the phosphorylation and localization of the protein connexin43 (Cx43), the main connexin in these cells (6), was also studied and compared to the effects observed on Cx43 in mouse primary keratinocytes.

As shown in Figure 3, TPA caused in both cell lines, as well as in primary keratinocytes, a hyperphosphorylation of Cx43 (i.e. a decreased intensity of the NP-band in concurrence with an increase in the intensity of the P_x-band). This is consistent with earlier findings (3,22). BoP however, decreased Cx43 phosphorylation in both primary keratinocytes and in the 3PC cells, as shown by a loss in intensity of the phosphorylated (P_x) band (Fig 3A & 3C). This agent however, increased Cx43 phosphorylation in CA3/7 cells (Fig.3B). This suggests that a change of Cx43 phosphorylation (either hypo- or hyper-phosphorylation) could affect gap junction functioning (compare effects TPA and BoP in the CA3/7 cell line). This is consistent with other reports associating inhibited GJIC with either hyperphosphorylation of Cx43 (4,22-24) or, in other cells types, with hypophosphorylation of Cx43 (25). These results also suggest that different tumor promoters may have different mechanisms to decrease GJIC, depending on the cell type used. By which mechanism tumor promoters change Cx43 phosphorylation is not exactly clear, because several protein kinases (PKA, PKC and Tyrosine kinase) are involved in the regulation of GJIC, leading to changes in Cx43 phosphorylation (for further references, see (5)).

An other possibility for the mechanisms of GJIC inhibition is the change in (amount or) localization of connexins. Figure 4 shows the effects of TPA and BoP on immunofluorescence staining of Cx43 in 3PC cells, CA3/7 cells and primary keratinocytes. In untreated cells Cx43 immunostaining is present on the cell membrane. In all cell types TPA caused a decrease in Cx43 immunostaining on the membrane.

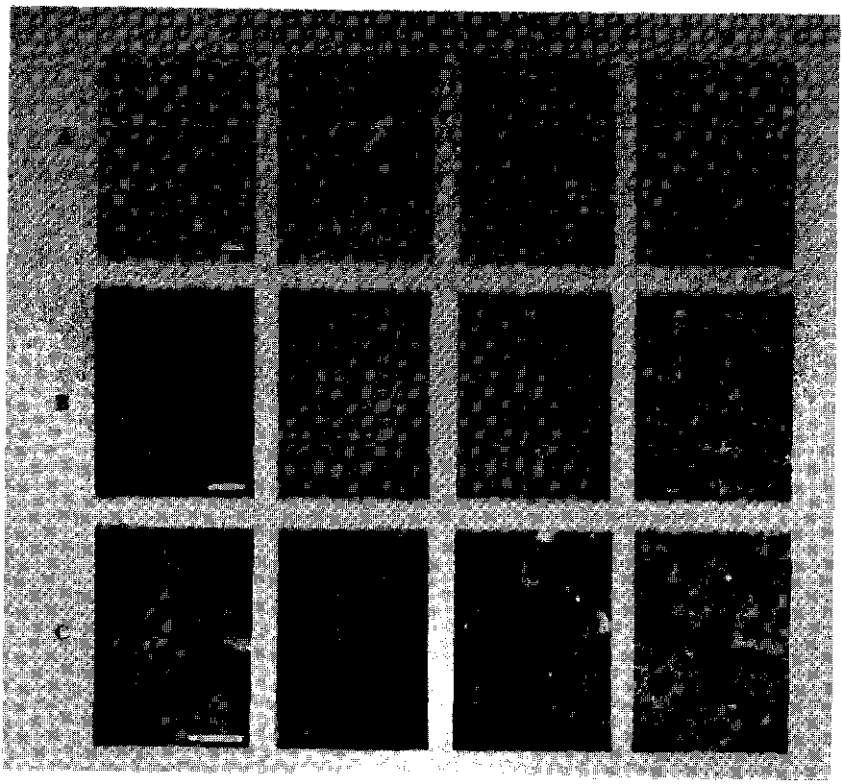

Fig 4. Immunostaining of connexin43 in 3PC cells (A), CA3/7 cells (B), and primary keratinocytes (C). Staining was performed with 1) untreated cells without first antibody (negative control), 2) untreated cells with first antibody (positive control), 3) TPA treated cells and 4) BoP treated cells. Bar is 25 μm in all series A, B, and C. Times of treatment and concentrations were as described in Fig.1. The films of pictures number 1-3, and A4 were exposed for a longer time, causing a non-specific autofluorescence of the cells in these pictures. Narrow arrow heads mark examples of staining on the membrane between cells, broad arrow heads mark examples of cytosolic staining in cells.

BoP strongly decreased Cx43 staining in 3PC cells and caused a Cx43 displacement from the membrane to the cytosol in CA3/7 cells and in primary keratinocytes. The results obtained with TPA are in agreement with other reports, showing that several tumor promoters (including TPA) can cause translocation of connexins from the

membrane to the cytosol or a decreased staining, although these changes do not always occur (3,4). A possible explanation for the fact that less intense Cx43 immunostaining is found (Figure 4; 3A, 3B, 3C) whereas the amount of Cx43 protein was not decreased by TPA (Figure 3) could be that individual connexons or small gap junction plaques give rise to less fluorochrome molecules per area unit, resulting in a very low fluorescence emission, which is below the visual limit. Taking into account the role of E-cadherin in the regulation of GJIC in mouse keratinocytes in general (6), the effects on GJIC may be mediated by the effect of the tumor promoters on E-cadherin. Further time sequence experiments could determine if the tumor promoter-induced effects on E-cadherin precede those on Cx43.

Overall these results show for the first time, that tumor promoters can decrease the quantity and membrane localization of the cell adhesion molecule E-cadherin. These results suggest that the loss of E-cadherin functioning could play a role in stages of tumor formation before tumor progression, which may lead to a decreased GJIC, which in turn is thought to play an important role in carcinogenesis (26).

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Chapter 7

Tumor promoters induce inhibition of gap junctional intercellular communication in mouse epidermal cells by affecting the localization of connexin43 and E-cadherin.

This chapter is based on the paper:
L.Jansen, M.Mesnil, J.Koeman, and W.Jongen (1996) Tumor promoters induce inhibition of gap junctional intercellular communication in mouse epidermal cells by affecting the localization of connexin43 and E-cadherin. Environm.Toxicol.Pharmac., 1, 185-192.

Abstract

The molecular and histological effects of tumor promoters on gap junctional intercellular communication (GJIC) were studied in three mouse epidermal cell types, representing different stages of tumor formation. GJIC was inhibited by most of the studied compounds (L-ethionine, d-limonene, o-anisidine, clofibrate, Aroclor 1260 and 1,1'-(2,2,2-trichloroethylidene)bis[4-chlorobenzene] (DDT)) except NaF and phenobarbital (PB). Whatever their effect on GJIC, most of the studied compounds increased the phosphorylation state of the gap junction protein expressed in these cells, connexin43 (Cx43), as shown by Western analysis. All agents with GJIC inhibiting capacity changed the intensity of the immunofluorescent staining of Cx43 on the membrane of the cells, whereas NaF and PB had no effect on Cx43 immunostaining. No association could be found between the type of change in Cx43 localization (changed membrane- and/or cytosolic staining) and Cx43 phosphorylation or GJIC inhibition. Because the cell adhesion molecule E-cadherin also regulates GJIC, the effects of tumor promoters on E-cadherin protein and -localization were studied. No quantitative change could be observed in E-cadherin protein content of cells treated with any of the selected agents. However, all agents which decreased GJIC, affected E-cadherin immunostaining of the membrane, while PB and NaF had no effect. These results show that an association exists between inhibition of GJIC and localization of both connexin43- and E-cadherin protein, but not with Cx43 phosphorylation.

Introduction

The most commonly accepted theory of chemically induced carcinogenesis, is the multistage concept. The initiation stage, in which cells undergo one or more DNA mutations, is followed by a promotion stage, in which cells undergo clonal expansion to form premalignant lesions (1,2). Inhibition of gap junctional intercellular communication (GJIC) has been postulated to be one of the important events which take place during the promotional stage. Indeed, it has been shown in vitro (3) and in vivo (4) that a large proportion of tumor promoters decrease GJIC as recently reviewed by Trosko and Chang (5) and by Budunova and Williams (6). Several other lines of evidence suggest a role for inhibition of GJIC during the process of cell transformation or tumor formation. For instance, a lack of GJIC between transformed and non-transformed cells has been reported by several groups on different cell types (7-9).

The precise mechanisms by which GJIC is regulated in particular by tumor promoters are not known. Several groups suggest a role for modulation of gap junction

protein (connexin) phosphorylation in the regulation of GJIC (10-13). Indeed, hyperphosphorylation as well as hypophosphorylation of gap junction proteins have been reported in vitro as well as in vivo, when GJIC was inhibited by tumor promoters (4,14,15). The type of effect of a tumor promoter on connexin phosphorylation may however be cell type specific, and/or compound specific (16-18). Apart from the level of phosphorylation, GJIC inhibition has also been associated with translocation of the connexin proteins from the cell-cell contact areas (gap junction plaques) to the cytosol. This has been reported for some tumor promoters, like TPA and DDT (3,4,16-18). It is however still unclear, how connexin phosphorylation and connexin location are associated with modulation of intercellular communication level. If this relationship exists, analysis of connexin phosphorylation and connexin localization could be used as parameters to predict effects of compounds on GJIC in vivo.

In mouse keratinocyte cell lines, the presence of the cell adhesion molecule E-cadherin on the membrane is essential for GJIC (19). In addition, the tumor promoter TPA has been shown to decrease cell assembly of primary keratinocytes (17). Furthermore, in a recent study, we demonstrated the effects of the tumor promoters benzoyl peroxide and TPA on the amount and location of E-cadherin in the mouse epidermal cell lines 3PC and CA3/7 (20). This suggests a direct association between the level of E-cadherin and inhibition of GJIC.

Previously, we showed differences in susceptibility for the inhibition of GJIC by tumor promoters of cell types representing different stages of tumor formation (21). In this study, we tried to get a better insight into factors determining these differences, by using histological and molecular analysis of effects by which tumor promoters affect GJIC in initiated (3PC) cells, carcinoma (CA3/7) cells and in primary keratinocytes. We determined the effects of tumor promoting agents on the phosphorylation- as well as the location of the gap junction protein connexin43 in all cell types. Furthermore, the effects of tumor promoting agents on the amount and localization of the cell adhesion molecule E-cadherin in both cell lines were also studied.

Results

First we determined the effect of the selected agents on GJIC in primary mouse keratinocytes and in the two mouse epidermal cell lines 3PC and CA3/7 (Table 1). The cell line 3PC was overall slightly more sensitive for inhibition of GJIC by tumor promoters compared to the cell line CA3/7. The susceptibility of primary keratinocytes to inhibition of GJIC compared to the 3PC cell line was variable, depending on the

Agent (μg/ml)	TIME (min)	Primary Keratinocytes	3PC (initiated cells)	CA3/7 (carcinoma cells)
Aroclor 1260 (200)	90	70	87	60
DDT (5)	90	48	58	32
PB (1000)	90	2 *	-5 *	4 *
Anisidin (1848)	90	55	62	46
Clofibrate (51)	70	nt	85	58
Ethionin (408)	90	-20 *	53	31
Limonene (136)	70	12 *	78	57
NaF (2100)	90	nt	3 *	12 *

Table 1: Percentage of inhibition of GJIC in different cell types after exposure to several tumor promoters or the negative control NaF. Negative numbers represent stimulation instead of inhibition of GJIC. The number of recipient cells under control conditions (18, 30 and 25 for respectively prim.ker., 3PC and CA3/7 cells) was not changed by the solvents used. *= not significantly different from control GJIC.

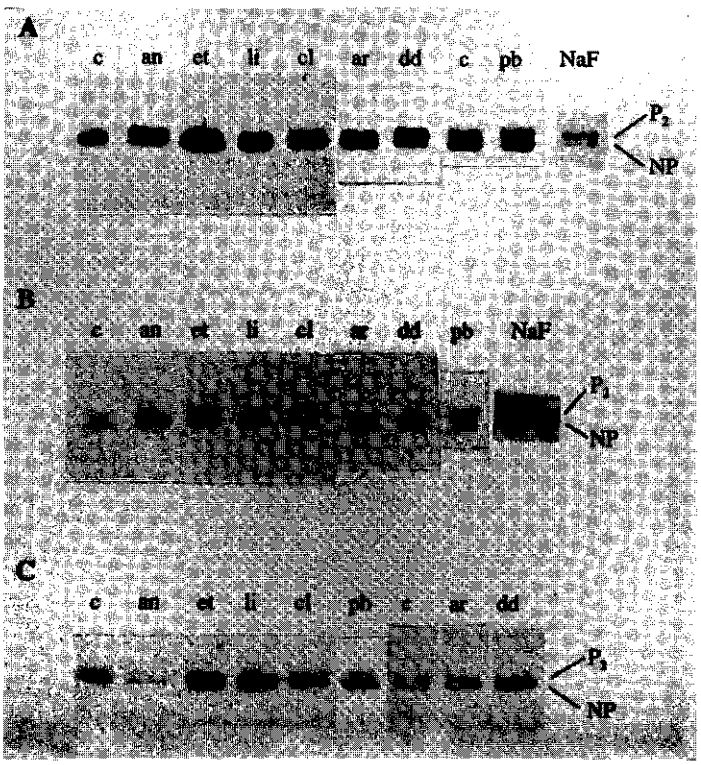

Figure 1. Western blot analysis of connexin43 in 3PC cells (A), CA3/7 cells (B) and primary keratinocytes (C). c= control (untreated) cells, an= anisidin, et= ethionin, li= limonene, cl= clofibrate, ar= Aroclor 1260, dd= DDT, NaF= NaF, and pb= PB.

agent used. NaF and PB did not affect GJIC in the cell lines 3PC and CA3/7 and were therefore used as negative controls in the molecular and histological assays.

Directly after exposure of the cells to the tumor promoter, the proteins were extracted and Cx43 phosphorylation was analysed by Western analysis. As shown for 3PC cells in Fig.1A, all selected agents, except for PB, increased the amount of phosphorylated Cx43 (an increased intensity of the double phosphorylated band (P_2) in concurrence with a decreased intensity of the non-phosphorylated (NP) band). PB had no effect on Cx43 phosphorylation in this cell line. Except for clofibrate which did not change Cx43 phosphorylation, all other agents (including NaF) increased Cx43 phosphorylation in CA3/7 cells (Fig 1B). In Fig 1C is shown, that in primary keratinocytes PB, clofibrate, ethionine, limonene and anisidine increased Cx43 phosphorylation, whereas anisidine also strongly decreased the amount of this gap junction protein. Aroclor 1260 and DDT had no effect on Cx43 phosphorylation in these cells. In Table 2 the results from the Western analysis are expressed as the ratio of the percentage phosphorylated Cx43 and the percentage phosphorylated Cx43 under control conditions. These results suggest that a decreased GJIC is associated with hyperphosphorylation of Cx43. However, NaF and PB treatments, which did not affect GJIC, also caused hyperphosphorylation of Cx43 in the different cell types.

	(% phosphorylated Cx43) / (% phosphorylated Cx43 control)		
Agent	3PC	CA3/7	Prim. Keratinocytes
DDT	1.75	1.55	1.06
Aroclor1260	1.46	1.54	1.06
o-Anisidin	2.14	1.25	1.55
Clofibrate	2.04	1.18	1.52
L-Ethionine	1.83	1.06	1.38
d-Limonene	2.07	0.79	1.42
Phenobarbital	1.24	1.61	1.14
NaF	1.79	1.48	nt

Table 2: The ratio of the percentage phosphorylated Cx43 in exposed cells and the percentage phosphorylated Cx43 under control conditions. The control % of phosphorylated Cx43 were respectively 38%; 33%, and 31% for 3PC cells, CA3/7 cells, and primary keratinocytes. nt = not tested.

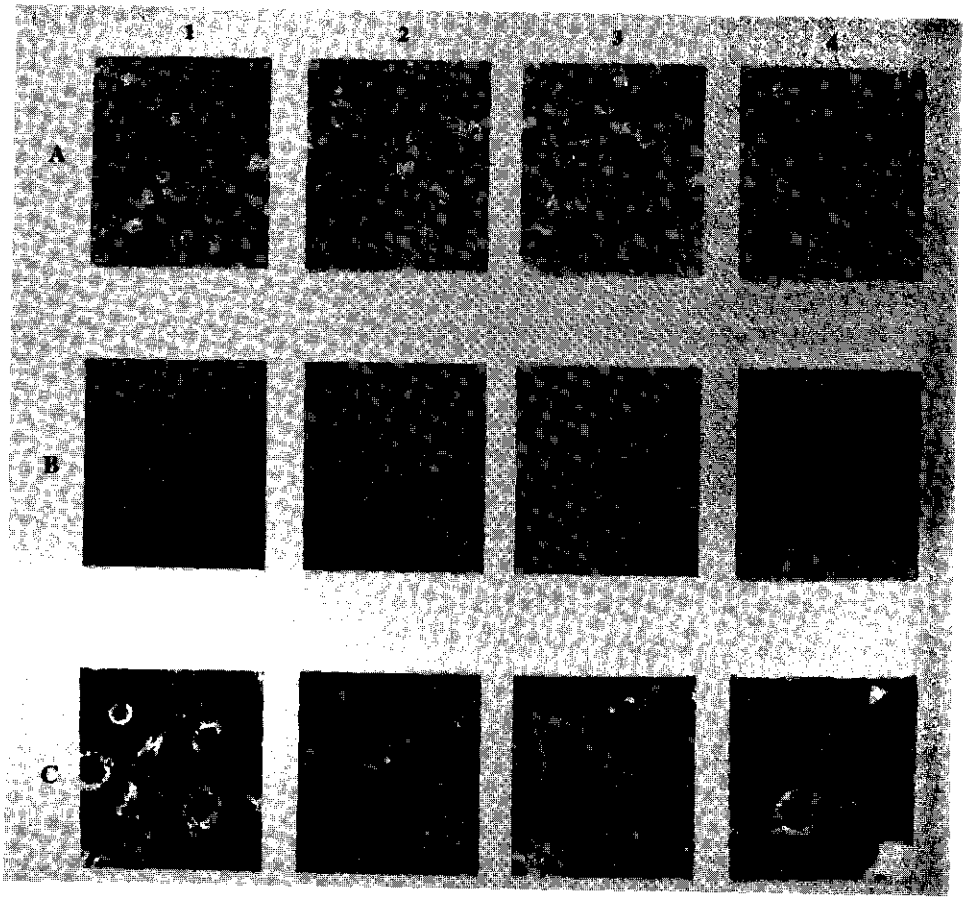

Figure 2. Immunostaining of Cx43 in 3PC cells (A), CA3/7 cells (B), and primary keratinocytes (C). Picture 1: control cells, same staining was found with NaF and PB treated cells. Picture 2: slightly decreased membrane staining of Cx43. Picture 3: strongly decreased staining of Cx43 on the membrane, in concurrence with increased cytosolic staining of Cx43. Picture 4: Strongly decreased Cx43 immunostaining, without increased cytosolic Cx43 staining. Narrow arrow heads mark examples of staining on the membrane between cells, broad arrow heads mark examples of cytosolic staining in cells. Bar = 20 μm in every serie of pictures.

We also examined the effect of these tumor promoters on the location of Cx43 in the cells. As shown in Fig. 2A, all tumor promoters with GJIC inhibiting capacity caused a decreased immunostaining of Cx43 on the cell membranes of 3PC cells. Limonene slightly decreased the membrane immunostaining of Cx43 in 3PC cells, as represented by Fig.2A2. Aroclor 1260, DDT and anisidine caused a slight change in immunofluorescence from the membrane to the cytosol (Fig.2A3), whereas clofibrate and ethionin strongly decreased the intensity of Cx43 immunostaining in 3PC cells. In the cell line CA3/7 (Fig 2B) both Aroclor1260 and clofibrate caused an increase of immunofluorescence in the cytosol in concurrence with a decreased immunofluorescence on the membrane (Fig.2B3), whereas the other agents strongly (limonene, Fig.2B4) or moderately (DDT, anisidine, ethionin, Fig.2B2) decreased the membrane Cx43 staining. Both NaF and PB had no effect on levels of Cx43 staining in both cell lines 3PC and CA3/7. In primary keratinocytes (Fig.2C), only Aroclor 1260 and DDT caused a changed immunofluorescence from the membrane to the cytosol (Fig.2C3). In these cells, ethionin, limonene and anisidine slightly decreased the membrane bound immunofluorescence (Fig.2C2), whereas clofibrate strongly decreased the Cx43 immunostaining (Fig.2C4). These results suggest that, besides phosphorylation of the gap junction protein, also the localization of this protein can be changed by tumor promoters.

Because, in mouse epidermal cells, GJIC was shown to be also regulated by the cell adhesion molecule E-cadherin (Jongen et al., 1991), and the tumor promoters TPA and benzoyl peroxide have been shown to decrease the amount of E-cadherin and/or the localization of this molecule (Jansen et al, submitted), we studied the effects of the selected agents on the quantity and localization of E-cadherin in both cell lines. Figure 3 shows that the amount of E-cadherin was not obviously decreased in the cell line CA3/7 when the cells were treated with the selected agents. The same results were obtained with the cell line 3PC (data not shown).

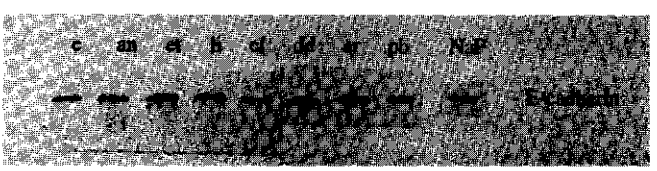

Figure 3. Western blot analysis of E-cadherin in CA3/7 cells. c = control (untreated) cells, an = anisidin, et = ethionin, li=limonene, cl = clofibrate, dd = DDT, ar = Aroclor 1260, and PB = phenobarbital.

Although the quantity of E-cadherin was not changed, its functionality (i.e.localization) may be affected by tumor promoters. This is shown in Fig. 4, in which E-cadherin immunostaining results of both cell lines are presented. In 3PC cells, clofibrate and limonene strongly decreased the membrane-bound immunofluorescence in concurrence with an increased staining around the nucleus (Fig.4A3). Aroclor 1260, DDT, ethionine, and anisidine caused translocation of E-cadherin from the membrane to the

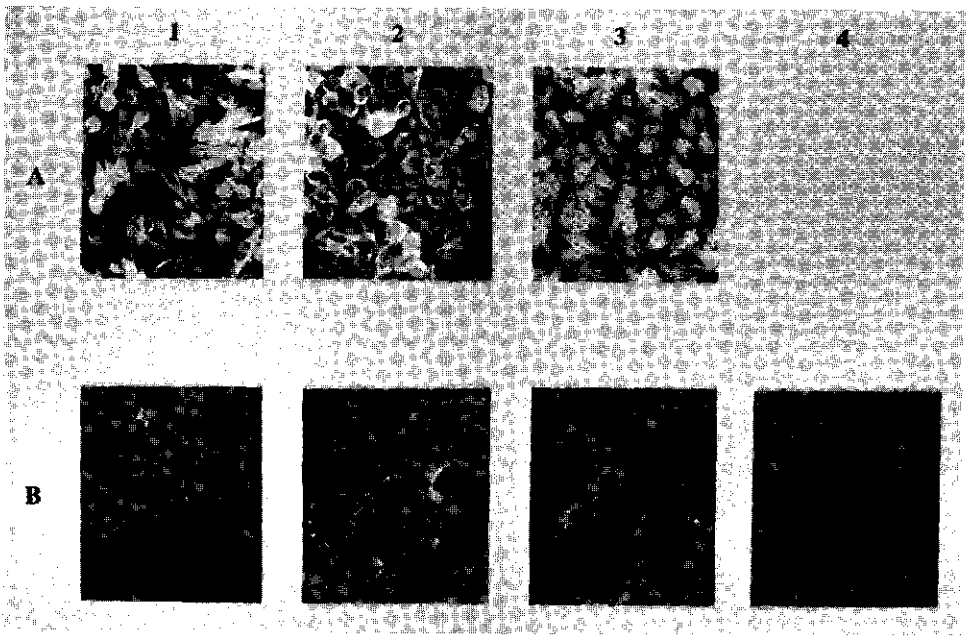

Figure 4. Immunostaining of E-cadherin in 3PC cells (A) and CA3/7 cells (B). Picture 1: control cells, same staining was found with NaF and PB treated cells. Picture 2: slightly decreased membrane staining of E-cadherin with (3PC) or without (CA3/7) increased cytosolic staining of E-cadherin. Picture 3: strongly decreased staining of E-cadherin on the membrane, without increased cytosolic staining. Picture 4: Very strong decrease of E-cadherin immunostaining. Narrow arrow heads mark examples of staining on the membrane between cells, broad arrow heads mark examples of cytosolic staining in cells. Bar = 20 μm in every serie of pictures.

cytosol to a lesser extent (Fig.4A2). In CA3/7 cells anisidin, ethionin and limonene weakly decreased E-cadherin bound membrane immunofluorescence, whereas DDT had a much stronger effect (Fig.4B2 resp. 4B4). Clofibrate and aroclor 1260 had an intermediate effect in these cells (Fig.4B3). In CA3/7 cells no cytosolic staining was observed. The negative controls, NaF and PB, did not affect E-cadherin immunostaining in the two cell lines.

Discussion

Inhibition of intercellular communication via gap junctions by tumor promoters has been shown both in vivo (4) and in vitro (3,6) to be an important event in the process of tumor formation. Since several tumor promoters are known to decrease GJIC in association with a changed level of phosphorylation of gap junction proteins (connexins) (14,15), we studied the effects of several agents on GJIC and on the phosphorylation of the major connexin expressed in the mouse epidermal cells we used (Cx43) (21). Previously, it was shown that differences occur in the susceptibility of cells for inhibition of GJIC by tumor promoters (22). Therefore, we compared the effects of exposure to tumor promoters on Cx43 phosphorylation in these cells. Fig 1 shows that most agents with GJIC inhibiting capacity caused an increase in phosphorylated Cx43 protein in concurrence with a decreased intensity of the non-phosphorylated Cx43 band (NP), in all three cell types. However, an increase in Cx43 phosphorylation is not strictly associated with a decreased GJIC, since agents with no effect on GJIC could also increase Cx43 phosphorylation (ethionine and limonene in Fig 1A, NaF in Fig 1B and 1C, PB in Fig 1A and 1C). Furthermore, GJIC can be affected without a change in Cx43-phosphorylation as shown by DDT and Aroclor 1260 in primary keratinocytes, as well as clofibrate in CA3/7 cells. Recently we found that benzoyl peroxide (BoP) treatment caused dephosphorylation of Cx43 protein in primary keratinocytes and in 3PC cells, whereas Cx43 was more phosphorylated in BoP treated CA3/7 cells (20). In addition TPA hyperphosphorylated Cx43 in all three cell types. All these results suggest that within one cell type, inhibition of GJIC can not be tightly associated with one explicit effect on Cx43 phosphorylation. These results also show that the effect of tumor promoters on Cx43 phosphorylation may vary, depending on the cell type used. This is consistent with the reports of other groups, that TPA has differing effects on Cx43 phosphorylation, depending on the cell type used (4,16-18).

GJIC could also be inhibited by changes in the localization of connexins. As

shown in Fig.2, all tumor promoters decreased the intensity of immunocytochemical Cx43 staining on the cell membrane. The effects of the agents used were not consistent in all three cell types. Exposure to some agents caused a decrease in membrane-associated Cx43-linked immunostaining with a concurrent increase in cytosolic staining, whereas with other agents only membrane associated staining was decreased. A previous study with mouse epidermal cell lines showed that inhibition of protein synthesis by cyclohexidine did not affect the extracellular calcium mediated increase of GJIC (19). This suggests that the turnover of Cx43 in these cells is not as high as shown for Cx26 in hepatocytes (23) and for Cx43 in cardiomyocytes (24). Because the total amount of Cx43 protein per mg of total protein was not affected by exposure of the cells to the tumor promoters (Fig 1), the decreased intensity of membrane Cx43 immunofluorescence, without an increased cytosolic staining, suggests a dispersion of the Cx43 protein from the plaques over the membrane, resulting in a fluorescent intensity below the visual detection limit.

In a former study we reported a decreased level of Cx43 immunostaining after TPA treatment of the same cells as used here (20). BoP changed the location of the Cx43-linked immunostaining. Several other reports showed a decreased or translocated Cx43 after exposure of cells to PCB (4), TPA (25-27), PB (4) and DDT (18,25), although these changes do not always occur (16,18). These results, together with the findings presented in this paper, show that Cx43 phosphorylation, as well as the cytoplasmic location of Cx43 are both dependent on the cell type and on the compound used. The effects on Cx43 immunostaining could not be linked with effects on Cx43 phosphorylation as tested by Western analysis, nor with effects on GJIC (as shown for 3PC cells in Table 3). It should be noted however, that all agents which affected GJIC, changed the location of Cx43 as tested by immunofluorescence.

Because E-cadherin has been shown to regulate GJIC in mouse keratinocytes (19), and E-cadherin amount and/or localization of E-cadherin can be changed by exposure of mouse keratinocytes to tumor promoters, we studied the effects of the selected agents on the amount and localization of E-cadherin in the two cell lines 3PC and CA3/7. All selected compounds did not affect the amount of E-cadherin as determined by Western analysis (Fig 3.). This suggests that the decreased amount of this cell adhesion molecule after treatment of the cells with BoP is an effect specific for this compound.

Agent (µg/ml)	Cx43 $ phosphorylation	Cx43 * localization	GJIC ^	E-cadherin * localization
Aroclor 1260 (200)	↑	→c	↓↓	→c
DDT (5)	↑	→c	↓↓	→c
PB (1000)	=	=	=	=
Anisidin (1848)	↑	→c	↓↓	→c
Clofibrate (51)	↑	↓↓	↓↓	↓↓
Ethionin (408)	↑	↓↓	↓↓	→c
Limonene (136)	↑	↓	↓↓	↓↓
NaF (2100)	↑	=	=	=
TPA (2)+	↑	↓	↓	→c
BoP (40)+	↓	↓↓	↓↓	↓↓↓

Table 3: Summary of the effects of the tumor promoters we tested on GJIC, on Cx43 phosphorylation and on immunostaining of Cx43 and E-cadherin in 3PC cells. $: symbols (↓, ↑, =) represent a decrease, an increase or an unchanged phosphorylation respectively. *: symbols represent a slightly (↓), a strongly (↓↓) or unchanged (=) staining, or a translocated staining to the cytosol (→c). ^: symbols represent level of inhibition of GJIC = (0-10%), ↓ (10-50%) and ↓↓ (50-100%). +: results from Jansen et al, submitted.

The change in localization of E-cadherin caused by the selected tumor promoting agents, as shown in Fig.4, is a further support of the finding that tumor promoting agents can affect the function of this cell adhesion molecule (20). As for Cx43 immonostaining, no association can be found between the degree of GJIC inhibition and the type of changed E-cadherin immunostaining (Table 3). The found results were however specific for the tested compounds with GJIC inhibiting capacity, because NaF and PB (only decreasing GJIC in 3PC cells after metabolic activation, (21)) did not affect E-cadherin localization in both cell lines.

By which mechanism tumor promoters can change the localization of both connexin43 and E-cadherin in these cells is unknown. (De)Activation of protein kinases could play a role in these mechanisms, as well in the mechanisms of GJIC inhibition, because the association between the activation of several protein kinases and the inhibition of GJIC, as well as the translocation of connexin43 has been observed (16,18,27-29). ß-catenin, a E-cadherin binding molecule with E-cadherin regulating capacity (30,31), has also been shown to loose functioning when phosphorylated on tyrosine (32,33). Furthermore it would be interesting to study time related tumor promoter induced changes in E-cadherin immunofluorescence and Cx43 immunofluorescence. This could give more information about the possibility that changes in

E-cadherin immunofluorescence are proceeding changes in Cx43 immunofluorescence. If this is the case, it would be in agreement with the findings that E-cadherin can regulate GJIC in mouse epidermal cells (19).

To determine whether any relationship exists between GJIC, Cx43 phosphorylation and Cx43 localization, the phosphorylation of Cx43 should be studied in more detail. For instance oncogene activation was shown to lead to inhibition of GJIC in concurrence with phosphorylation of Cx43 on tyrosine residues, and exposure of rat liver epithelial cells to epidermal growth factor induced inhibition of GJIC and Cx43 phosphorylation on serine residues (34,35). Phosphorylation on specific amino acid residues of Cx43 might give more information about the specificity of tumor promoter-induced Cx43 phosphorylation, and its relation with GJIC inhibition.

Overall these results show that the level of Cx43 protein phosphorylation is not predictive for the effect on GJIC. Furthermore, an effect on GJIC is associated with a change in Cx43- and E-cadherin linked immunostaining, although the effect on the immunostaining of these proteins is agent- as well as cell type specific.

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Chapter 8

The role of calcium in tumor promoter-mediated inhibition of gap junctional intercellular communication

This paper is based on the paper:
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Abstract

The effect of several tumor promoters (12-*O*-tetradecanoyl-phorbol-13-acetate (TPA); 1,1'-(2,2,2-trichloroethylidene)bis[4-chlorobenzene] (DDT); Aroclor1260; L-ethionine; d-limonene; o-anisidine, and clofibrate) on the inhibition of gap junctional intercellular communication (GJIC) and intracellular calcium concentration ($[Ca^{2+}]_i$) were studied in a cell line consisting of initiated cells (3PC) and a carcinoma-derived cell line (CA3/7). In addition, the effect of different extracellular calcium concentrations ($[Ca^{2+}]_e$) on the effects of tumor promoters on both GJIC and $[Ca^{2+}]_i$ were studied, using the same tumor promoters. Agents with GJIC inhibiting capacity also increased $[Ca^{2+}]_i$ in both cell lines. However, the increase of $[Ca^{2+}]_i$ did not (always) precede GJIC inhibition, suggesting that the effect of tumor promoters on GJIC is not directly related to their effect on $[Ca^{2+}]_i$. This is supported by the finding that tumor promoter-mediated inhibition of GJIC in the 3PC cell line did not differ between low (0.05 mM) and high (1.20 mM) Ca^{2+}_e conditions, while differing effects on $[Ca^{2+}]_i$ were found.

The differences in susceptibility between the two cell lines for inhibition of GJIC by tumor promoters could not be explained by the effects of the used agents on the $[Ca^{2+}]_i$ in both cell types. These results suggest that tumor promoters can inhibit GJIC and change $[Ca^{2+}]_i$, but that there is no direct relationship between these two processes.

Introduction

It has been hypothesised that in the multistep process of tumor formation, a decreased gap junctional intercellular communication (GJIC) could give genetically altered (initiated) cells the possibility for clonal expansion (1,2). Gap junctions are intercellular channels, permitting small metabolites, ions and second messengers to pass to neighbouring cells. The channels are made of juxtaposed connexons crossing the membrane of two adjacent cells. Each connexon is formed by six protein subunits called connexins (3,4). How the functioning of connexons is regulated is still unknown but several groups have suggested that changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) are involved in the regulation of GJIC (5-7). Furthermore two tumor promoters which decreased GJIC, raised $[Ca^{2+}]_i$ (8). This changed $[Ca^{2+}]_i$ could (de)activate GJIC modulating molecules or enzymes, like calmodulin or Ca^{2+}/calmodulin dependent protein kinases. Recently we showed the involvement of both $[Ca^{2+}]_i$ and calmodulin (CaM) in the regulation of GJIC in a cell line consisting of initiated cells (3PC) and a carcinoma derived cell line (CA3/7) (Jansen et al.,

submitted). In 3PC cells under low extracellular calcium (Ca^{2+}_e) conditions (0.05 mM), GJIC was inhibited by the Ca^{2+}_i chelator EGTA-AM, while an increase of $[Ca^{2+}]_i$ did not change the high level of GJIC. In CA3/7 cells cultured under low Ca^{2+}_e conditions, the low GJIC level could be increased by raising $[Ca^{2+}]_i$. Under high $[Ca^{2+}]_e$ conditions (1.2 mM), both cell types had a high GJIC level, which remained unaffected while $[Ca^{2+}]_i$ was increased by calcium ionophores or decreased by EGTA-AM. If calcium is to play a role in the tumor promoter-mediated inhibition of GJIC, these results suggest an decreased $[Ca^{2+}]_i$ as a results of exposure to tumor promoters. However, since the $[Ca^{2+}]_e$ is 500 to 10^4 times higher (for low and high Ca^{2+} medium) then $[Ca^{2+}]_i$, a decrease of $[Ca^{2+}]_i$ is most unlikely to occur, but a significant increase might be expected as a result of effects of tumor promoters on calcium transport over (plasma) membranes. That would agree with the results of other groups, describing an inhibition of GJIC when $[Ca^{2+}]_i$ was increased (7,9).

We recently demonstrated that both cell lines can be used for the detection of GJIC-inhibiting agents (10). To get more insight in the mechanism by which tumor promoters inhibit GJIC, we determined the effects of several tumor promoters on GJIC and on $[Ca^{2+}]_i$ in 3PC cells. In addition, the possible influence of $[Ca^{2+}]_e$ on the tumor promoter-mediated inhibition of GJIC and change of $[Ca^{2+}]_i$ were studied. Because the sensitivity of CA3/7 cells for the inhibition of GJIC by tumor promoters is different compared to 3PC cells (10), we also studied the effects of some tumor promoters on GJIC and the $[Ca^{2+}]_i$ in CA3/7 cells.

Results

Several compounds with a GJIC inhibiting capacity in a broad spectrum of cell types (11) were used. The GJIC level of 3PC cells under low (0.05 mM) Ca^{2+}_e conditions (mean = 30 communicating cells) remained unchanged during 90 minutes under these conditions, and was not changed either under high (1.20 mM) or Ca^{2+}_e-free conditions (data not shown). As shown in Figure 1A, TPA-mediated inhibition of GJIC in 3PC cells was comparable under low compared to high Ca^{2+}_e conditions. In Ca^{2+}-free PBS, the inhibition of GJIC was comparable to that under the other two Ca^{2+}_e conditions up to 45 minutes of exposure, and significantly stronger after 90 minutes of exposure. Under Ca^{2+}_e-free conditions, TPA exposure did not affect the intracellular calcium concentration ($[Ca^{2+}]_i$) (Fig.1B). TPA exposure under low Ca^{2+}_e conditions increased $[Ca^{2+}]_i$, whereas under high Ca^{2+}_e conditions, $[Ca^{2+}]_i$ was strongly increased. The polychlorobiphenyl mixture Aroclor1260 (200 µg/ml) decreased the GJIC level in 3PC

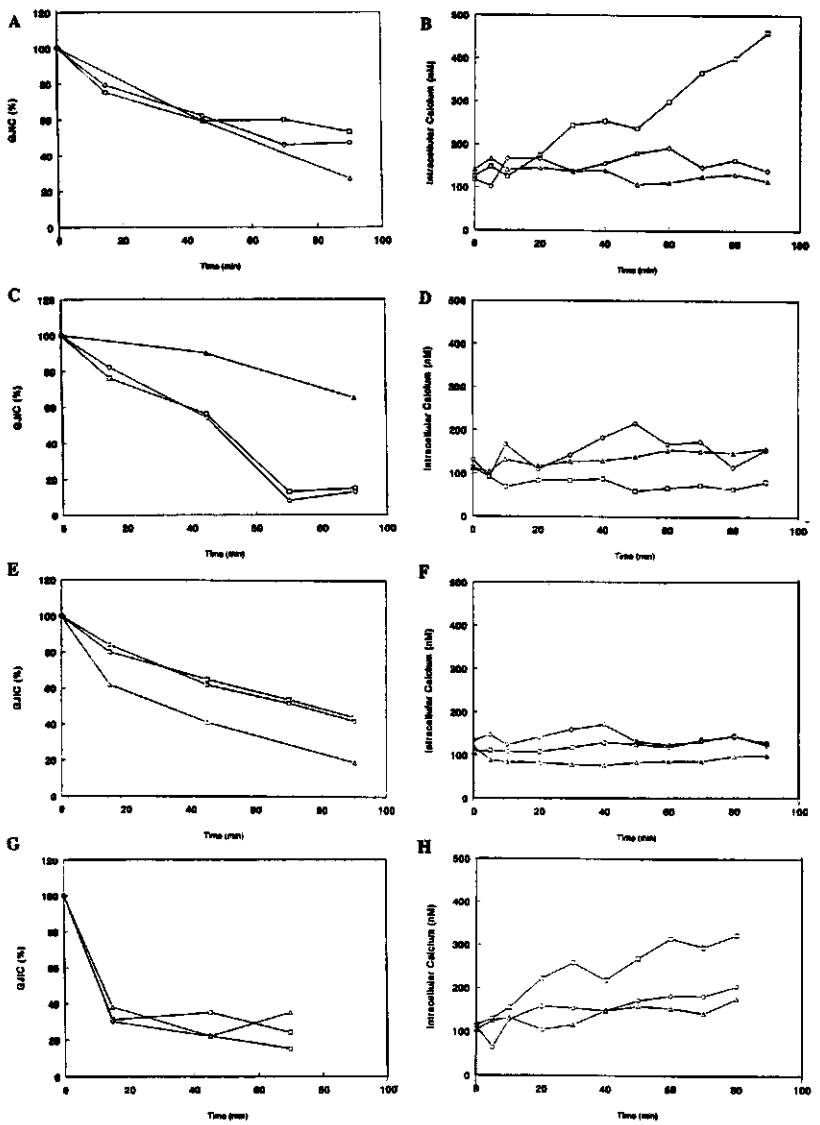

Figure 1: The effect of several agents on GJIC (A,C,E,G) and $[Ca^{2+}]_i$ (B,D,F,H) in 3PC cells under Ca^{2+}_e-free conditions (triangular symbols); low Ca^{2+}_e conditions (round symbols), and high Ca^{2+}_e conditions (square symbols). After determining the control level of GJIC (=100%) and $[Ca^{2+}]_i$, the cells were exposed to 4 µg/ml TPA (A,B); 200 µg/ml Aroclor1260 (C,D); 5 µg/ml DDT (E,F); or 51 µg/ml clofibrate (G,H), after which the effects on GJIC or $[Ca^{2+}]_i$ were studied.

cells comparably under low Ca^{2+}_e and high Ca^{2+}_e conditions (Fig.1C). In the absence of Ca^{2+}_e however, the GJIC level was less affected by exposure of the cells to Aroclor1260. Whereas under high Ca^{2+}_e conditions, $[Ca^{2+}]_i$ was not significantly changed during exposure, Aroclor1260 increased $[Ca^{2+}]_i$ in 3PC cells in Ca^{2+}-free medium and in low Ca^{2+} medium (Fig.1D). The DDT (5 µg/ml)-mediated inhibition of GJIC was comparable under low and high Ca^{2+}_e conditions, while in Ca^{2+}-free PBS the GJIC level was decreased stronger (Fig.1E). DDT induced only under low Ca^{2+}_e conditions a temporally significant increase of $[Ca^{2+}]_i$, while under the high Ca^{2+}_e conditions $[Ca^{2+}]_i$ remained unchanged, and under Ca^{2+}_e-free conditions, $[Ca^{2+}]_i$ was decreased (Fig.1F). Under all three Ca^{2+}_e conditions, the clofibrate (51 µg/ml)-induced inhibition of GJIC in 3PC cells was in the same order of magnitude (Fig.1G). The effects of clofibrate on $[Ca^{2+}]_i$ was however dependent on the Ca^{2+}_e conditions. Exposure of the cells under a higher Ca^{2+}_e condition resulted in a stronger increase of $[Ca^{2+}]_i$ (Fig.1H). The GJIC inhibiting capacity of anisidin, ethionine and limonene were recently shown in these cells and in primary keratinocytes (10). The effects of these agents on GJIC and $[Ca^{2+}]_i$ were studied in 3PC cells under low Ca^{2+}_e conditions (Fig.2). Both ethionine (408 µg/ml) and limonene (136 µg/ml) strongly decreased the GJIC level within 15 minutes of exposure (Fig.2A). Upon longer exposure times, the

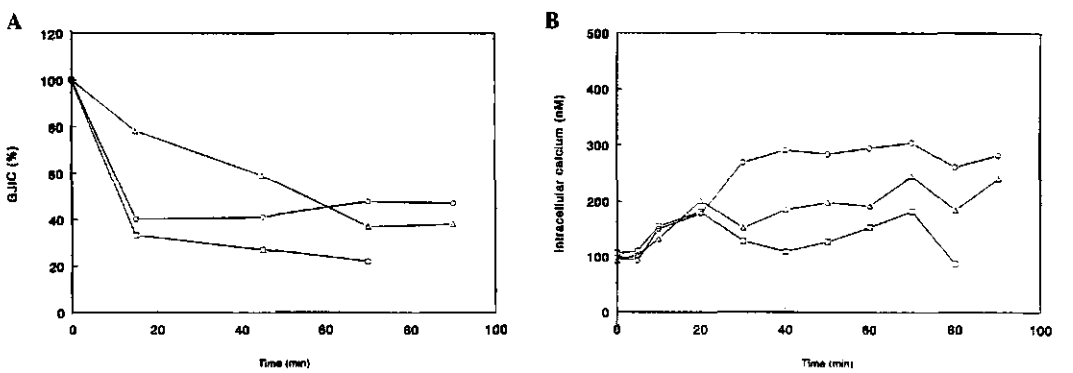

Figure 2: The effect of 1848 µg/ml anisidine (triangular symbols); 408 µg/ml ethionine (round symbols) or 136 µg/ml limonene (square symbols) on GJIC (A) and $[Ca^{2+}]_i$ (B) in 3PC cells under low Ca^{2+}_e conditions. The effects were studied as described in Fig.1 for other compounds.

GJIC level was further decreased in limonene treated cells, whereas in ethionine-treated cells the GJIC level remained the same. Exposure of the cells to anisidine (1848 µg/ml) resulted in a gradual decrease of GJIC after 70 and 90 minutes of exposures to a level comparable to limonene- and ethionine-treated cells. Exposure to all 3 compounds for 20 minutes resulted in an almost doubled $[Ca^{2+}]_i$ (Fig.2B). After 20 minutes of exposure, $[Ca^{2+}]_i$ raised further in anisidine- and ethionine-treated cells. In limonene-exposed cells, the $[Ca^{2+}]_i$ returned to the control level after 40 minutes of exposure. Then $[Ca^{2+}]_i$ raised again, after which $[Ca^{2+}]_i$ returned to the control level.

The carcinoma derived cell line (CA3/7) has a low GJIC level under low Ca^{2+}_e conditions compared to 3PC cells (12). When the $[Ca^{2+}]_e$ was increased to 1.20 mM, the GJIC level of CA3/7 cells raised to a level comparable to 3PC cells. The effect of several of the selected compounds on GJIC and $[Ca^{2+}]_i$ in CA3/7 cells were studied under high Ca^{2+}_e conditions, because of the high GJIC level of these cells under these conditions. Because the $[Ca^{2+}]_e$-mediated increase of GJIC in CA3/7 cells could be due to a Ca^{2+}_e-mediated increase of $[Ca^{2+}]_i$ (Jansen et al., submitted), the effects of a tumor promoter on GJIC and on $[Ca^{2+}]_i$ were also studied under low Ca^{2+}_e conditions if a tumor promoter increased $[Ca^{2+}]_i$ under high Ca^{2+}_e conditions.

As shown in Fig.3A, 2 µg/ml TPA and 200 µg/ml Aroclor1260 decreased the GJIC level in CA3/7 cells to the same degree. Under high Ca^{2+}_e conditions in CA3/7 cells, TPA induced a transient $[Ca^{2+}]_i$ increases (Fig.3B), while under low Ca^{2+}_e conditions $[Ca^{2+}]_i$ remained unchanged after TPA exposure (data not shown). Under high Ca^{2+}_e conditions, Aroclor1260 increased $[Ca^{2+}]_i$ significantly in CA3/7 cells from 30 minutes of exposure. Under low Ca^{2+}_e conditions, Aroclor1260 induced one $[Ca^{2+}]_i$ peak after 40 minutes and $[Ca^{2+}]_i$ was increased after 90 minutes of exposure. DDT (5 µg/ml) inhibited GJIC in CA3/7 cells up to 25% after 90 minutes of exposure (Fig.3C). In addition, DDT exposure of CA3/7 cells under high Ca^{2+}_e conditions increased $[Ca^{2+}]_i$ (Fig.3D), whereas under low Ca^{2+}_e conditions $[Ca^{2+}]_i$ remained unchanged (data not shown). GJIC between CA3/7 cells was stronger affected by 51 µg/ml clofibrate under high Ca^{2+}_e conditions compared to the effect of DDT. Clofibrate strongly increased $[Ca^{2+}]_i$ after 70 minutes of exposure of CA3/7 cells under low and high Ca^{2+}_e conditions. Because the Ca^{2+}_e-mediated increase of the GJIC level in CA3/7 cells could due to a $[Ca^{2+}]_e$-induced increase of $[Ca^{2+}]_i$ (Jansen et al., submitted), we studied the effect of a clofibrate-induced increase of $[Ca^{2+}]_i$ on the GJIC level in CA3/7 cells under those conditions. The percentage of remaining GJIC in CA3/7 cells under low Ca^{2+}_e conditions was however decreased by clofibrate to a comparable level as under high

Ca^{2+}_e conditions (Fig.3C).

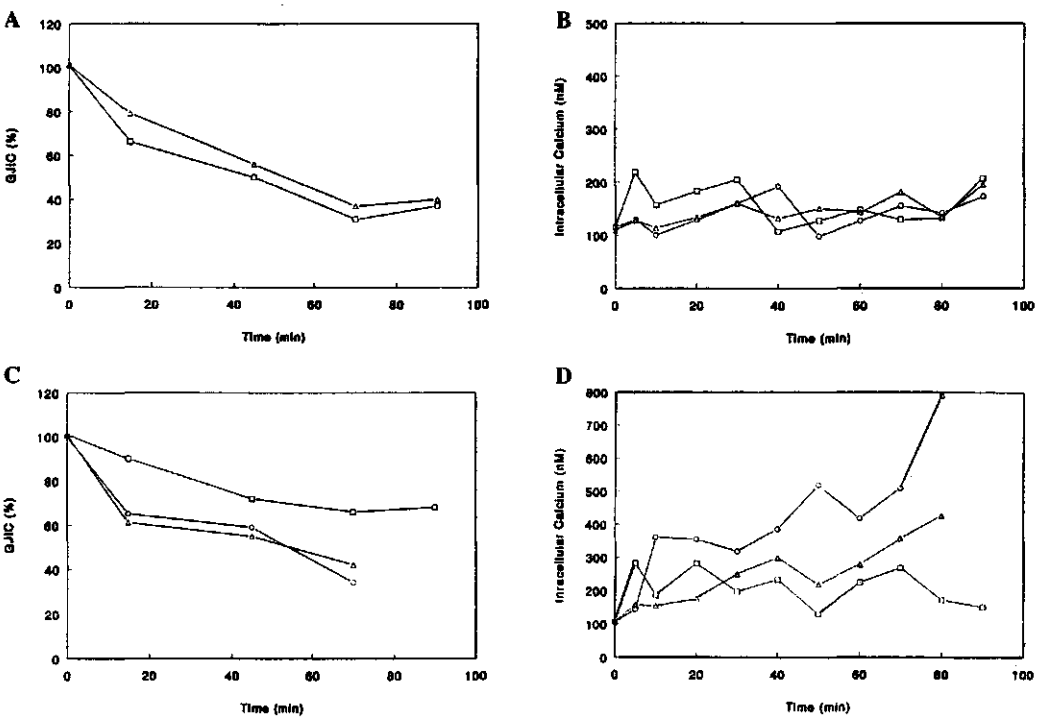

Figure 3: The effect of of several agents on GJIC (A,C) and $[Ca^{2+}]_i$ (B,D) in CA3/7 cells under low Ca^{2+}_e conditions (round symbols), and high Ca^{2+}_e conditions (square or triangular symbols). After determining the control level of GJIC (=100%) and $[Ca^{2+}]_i$, the cells were exposed to 4 μg/ml TPA (A,B; square symbols); 200 μg/ml Aroclor1260 (A,B; round or triangular symbols); 5 μg/ml DDT (C,D; square symbols); or 51 μg/ml clofibrate (C,D; round or triangular symbols), after which the effects on GJIC or $[Ca^{2+}]_i$ were studied.

Discussion

In this study we demonstrated that, in addition to an inhibitory effect on gap junctional intercellular communication, agents with tumor promoter capacity can also change intracellular Ca^{2+} levels. One of the most widely used tumor promoters, TPA, inhibited GJIC in 3PC cells to the same extent as in CA3/7 cells. In addition, TPA induced (transient) increases of $[Ca^{2+}]_i$ in both cell lines. The TPA-induced increased of $[Ca^{2+}]_i$

in both cell types appeared to be dependent on the $[Ca^{2+}]_e$. Under conditions in which the $[Ca^{2+}]_e$ was higher, the $[Ca^{2+}]_i$ increase was also higher. Several other groups have reported an effect of TPA on $[Ca^{2+}]_i$. In MDCK cells the $[Ca^{2+}]_i$ was doubled within 30 minutes of exposure of the cells to TPA-containing medium under high Ca^{2+}_e conditions (13). This $[Ca^{2+}]_i$ increase is comparable with the effect of TPA found in both CA3/7 cells and 3PC cells under high Ca^{2+}_e conditions. In V79 cells TPA was shown to increase the Ca^{2+} influx, and to inhibit its efflux (8). These results, together with our results presented here, demonstrate that TPA can increase $[Ca^{2+}]_i$ in various cell types. The induced $[Ca^{2+}]_i$ increase may be due to an increase of the activity of protein kinase C (PKC) by TPA (14). Indeed, PKC has been shown to play a role in the regulation of $[Ca^{2+}]_i$ in several cell types (6,15,16), which may be due to an effect on the Ca^{2+}-ATPase activity (17). However, the TPA-induced $[Ca^{2+}]_i$ change might also be the result of other non PKC-dependent mechanisms (8).

Aroclor1260-mediated inhibition of GJIC was significantly stronger in 3PC cells compared to CA3/7 cells after 70 minutes of exposure. It is interesting to note that in Ca^{2+}-free medium, the inhibition of GJIC in 3PC cells is much lower compared to the inhibition of GJIC in medium containing low or high $[Ca^{2+}]$. This suggests a role for Ca^{2+}_e in the Aroclor1260-mediated inhibition of GJIC. The effects on $[Ca^{2+}]_i$ in CA3/7 cells are comparable to those observed in 3PC under the same Ca^{2+}_e conditions. Aroclor1260 induced a (transient) $[Ca^{2+}]_i$ increase under Ca^{2+}_e-free (3PC) and under low Ca^{2+}_e conditions (CA3/7 and 3PC), but not under high Ca^{2+}_e conditions in both cell types. GJIC was however strongly decreased in both cell lines under high Ca^{2+}_e conditions, suggesting that the role of $[Ca^{2+}]_i$ in the Aroclor1260-mediated inhibition of GJIC is limited.

In contrast to the stimulating role of Ca^{2+}_e in the inhibition of GJIC in 3PC cells by Aroclor1260, DDT inhibited GJIC stronger in the same cell type under Ca^{2+}_e-free conditions compared to conditions in the presence of Ca^{2+}_e. These results suggests that different mechanisms are involved in the inhibition of GJIC by these two chlorinated hydrocarbons. Exposure of 3PC cells to DDT under Ca^{2+}_e-free conditions induced a strong inhibition of GJIC in concurrence with a decreased $[Ca^{2+}]_i$. DDT increased $[Ca^{2+}]_i$ only transiently under low Ca^{2+}_e conditions in 3PC cells, and under high Ca^{2+}_e conditions in CA3/7 cells. The inhibition of GJIC was significantly less in CA3/7 cells compared to 3PC cells. These differences can be explained by the effects of DDT on the increase of $[Ca^{2+}]_i$ under high Ca^{2+}_e conditions, which were stronger in CA3/7 cells compared to the effects in 3PC cells. This, together with the increased inhibition of

GJIC in 3PC cells under Ca^{2+}_e-free conditions, suggests a protecting mechanism of Ca^{2+}_i on the inhibition of GJIC by DDT.

3PC cells were more sensitive for the inhibition of GJIC by clofibrate compared to CA3/7 cells. Although inhibition of GJIC by clofibrate in both 3PC cells and CA3/7 cells was not dependent on the $[Ca^{2+}]_e$, the increase of $[Ca^{2+}]_i$ in 3PC cells was higher when $[Ca^{2+}]_e$ was higher. In CA3/7 cells, the $[Ca^{2+}]_i$ was stronger increased under low Ca^{2+}_e conditions compared to high Ca^{2+}_e conditions. This increased $[Ca^{2+}]_i$ under high Ca^{2+}_e conditions was comparable with the effect of clofibrate on $[Ca^{2+}]_i$ in 3PC cells under high Ca^{2+}_e conditions. The clofibrate-induced increase of $[Ca^{2+}]_i$ under low Ca^{2+}_e conditions was much stronger in CA3/7 cells compared to 3PC cells. The fact that in 3PC cells GJIC was decreased before significant changes of $[Ca^{2+}]_i$ were observed suggests that the role of $[Ca^{2+}]_i$ in the clofibrate-induced inhibition of GJIC is limited. The effect of clofibrate on both GJIC and $[Ca^{2+}]_i$ might be the result of reactive oxygen products produced in the (clofibrate-stimulated) peroxysomes (18).

The role of $[Ca^{2+}]_i$ in the inhibition of GJIC in 3PC cells by anisidine, limonene, and ethionine is not clear. In the first 20 minutes of exposure of 3PC cells to these agents the $[Ca^{2+}]_i$ was nearly doubled, while the GJIC was inhibited for 70% by ethionine and limonene, and only for 25% by anisidine. Furthermore, the changes in $[Ca^{2+}]_i$ induced by these 3 compounds show no relationship with the effects on GJIC during longer exposure times of 3PC cells to these agents. Possible mechanisms by which these compounds could inhibit GJIC or change $[Ca^{2+}]_i$ are still unknown.

All together these results show that tumor promoters have different effects on $[Ca^{2+}]_i$, and that these effects on $[Ca^{2+}]_i$ are not directly related to effects on GJIC, except for the effects of DDT in 3PC cells. The finding that tumor promoters have different effects on $[Ca^{2+}]_i$ is additional to the results of our recent study in which different effects of tumor promoters were found on Cx43 phosphorylation, Cx43 location, and E-cadherin location (19). These data point to agent specific mechanisms by which tumor promoters inhibit GJIC, not excluding the possibility that some tumor promoters might exert their effects by the same mechanism. The differences between 3PC cells and CA3/7 cells in the sensitivity for inhibition of GJIC by tumor promoters can not be explained by the effects of tumor promoters on $[Ca^{2+}]_i$ in both cell lines. These results also demonstrate that, under different Ca^{2+}_e conditions within one cell line, GJIC can be inhibited by tumor promoters to the same degree, while different effects on $[Ca^{2+}]_i$ are induced. This suggests that inhibition of GJIC and a change of the $[Ca^{2+}]_i$ could be two different, non relating effects of exposure of cells to a tumor

promoting agent. For instance, the activation of PKC by TPA could change the Ca^{2+}-ATPase activity and inhibit GJIC at the same time. Therefore, more research is needed to specify the possible role of calcium in tumor promoter-induced inhibition of GJIC.

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Chapter 9

Summary and concluding remarks

Summary and concluding remarks

Gap junctional intercellular communication (GJIC) plays an important role in the differentiation and growth of cells (1). Increasing evidence also suggests a role for inhibition of GJIC in the promotion phase of tumor formation (2-8). How the level of GJIC is regulated in normal cells, and if this regulation is changed during the process of tumor formation is however not clearly known yet.

In the chapters 3 and 4 the studies on the regulation of GJIC by (extracellular and intracellular) Ca^{2+}- and cAMP-dependent processes are described for a cell line consisting of initiated cells (3PC), and a carcinoma derived cell line (CA3/7). From these studies it can be concluded that differences exists between the regulation of GJIC in cells representing different stages in the process of tumor formation, and that the short-term regulation of GJIC by intracellular Ca^{2+} (Ca^{2+}_i) or by cAMP are different routes of regulation. Furthermore it can be concluded that the presence of cell adhesion molecule E-cadherin on the plasma membrane is a prerequested for a high GJIC level, but that E-cadherin is not involved in the short term regulation of GJIC by Ca^{2+}_i or cAMP.

The observed differences between 3PC cells and CA3/7 cells in the regulation of GJIC by intracellular signals suggest that during the process of tumor formation changes have occured in the Ca^{2+}_i- or calmodulin-dependent regulation of GJIC. These differences may be the result of differences in intracellular concentrations of regulating molecules (such as Ca^{2+}_i or CaM) or differences in the sensitivity of enzymes which are dependent of Ca^{2+}_i or CaM. These differences could result in a blocked intercellular communication between cell types representing different stages of tumor formation. For instance, if normal cells with a high GJIC level would come in contact with preneoplastic cells with different intracellular concentrations of calcium, a diffusion of calcium would appear at the moment intercellular gap junctions become functional between the two cell types. This diffusion of calcium will then lead to changed concentrations in the intracellular gap junction regions, which could result in mechanisms closing the gap junction to preserve the cellular homeostasis. Together this might lead to a quickly blocked GJIC between the normal and the preneoplastic cells, which could have a high homoloques GJIC level, as was shown between transformed and non-transformed Balb/c 3T3 cells (9).

Agents which decrease the level of GJIC (including tumor promoters) can play a role in the promotion phase of the process of tumor formation. Several test systems (based on the inhibition of GJIC) for the detection of agents with GJIC inhibiting

capacity exist (3,4). In these assays however, the target cells for tumor promoters in the process of tumor formation (i.e. initiated cells) are not used. From the work presented in chapter 5 it can be concluded that a cell line consisting of initiated mouse epidermal cells (3PC) is a good model to detect agents with GJIC-inhibiting capacity, and that these cells are more sensitive for inhibition of GJIC compared to carcinoma-derived cells. The sensitivity of primary keratinocytes compared to 3PC cells was varying and dependent on the agent used.

To determine if the differences between the cell types used for the detection of inhibition of GJIC (chapter 5) are attributable to differences in mechanisms which are thought to play a role in the regulation of GJIC (i.e. connexin amount, connexin phosphorylation, connexin location in the cell, E-cadherin amount, and E-cadherin location in the cell), we studied the effects of tumor promoters on these parameters in the different cell types (chapters 6 and 7). Because calcium plays a role in the regulation of GJIC (chapter 3), we also studied the effect of tumor promoters on Ca^{2+}_i and the role of Ca^{2+}_e on these effects (chapter 8). From these studies, the following conclusions can be drawn: 1) The mechanisms involved in the inhibition of GJIC by tumor promoters are agent- and cell type-dependent; 2) The observed differences in the susceptibility of cells for the inhibition of GJIC by tumor promoters can not be associated with effects of the studied agents on one of the studied parameters; and 3) tumor promoters can change $[Ca^{2+}]_i$, but these changes are not associated with inhibition of GJIC.

In addition to the results of the studies on the regulation of GJIC (chapters 3 and 4), the studies with tumor promoters showed that, in the mouse epidermal cells used in these studies, only a decreased immunostaining of Cx43 on the plasma membranes of cells can be related to a decreased GJIC level. However, it must be noted that specific amino acid analysis of Cx43 could finally demonstrate if a relationship between inhibition of GJIC and Cx43 phosphorylation exists et all.

The mechanisms regulating the transport of connexins to and from the membrane, as well as the mechanisms involved in the formation of connexons are yet unknown. Because several tumor promoters inhibited GJIC in addition to delocation of both Cx43 and E-cadherin, it should be interesting to study the mechanisms involved in the assembly of gap junction proteins or cell adhesion molecules in the plasma membrane. Such study could lead to a better understanding of the mechanisms involved in (chemical-induced) inhibition of GJIC. A study on the time-related changes in tumor promoter-induced Cx43 immunostaining and E-cadherin immunostaining

could give more insight in the role of E-cadherin in the regulation of GJIC. For such a study, also cells transfected with E-cadherin or a connexin could be used.

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Chapter 10

Samenvatting en slotopmerkingen

Samenvatting en slotopmerkingen

Intercellulaire communicatie via gap junctions (GJIC) speelt een belangrijke rol in de differentiatie van cellen (1). Aanvullend bewijs suggereert een rol voor remming van GJIC in de promotie fase van tumor vorming (2-8). Hoe het GJIC nivo in normale cellen is gereguleerd, en of deze regulatie verandert gedurende het proces van tumor vorming is echter nog onbekend.

In de hoofdstukken 3 en 4 van dit proefschrift zijn de studies naar de regulatie van GJIC door (intracellulair en extracellulair) Ca^{2+}- en cAMP-afhankelijke processen beschreven voor een cellijn afkomstig van geïnitieerde epidermale cellen van een muis (3PC), en van een cellijn afkomstig van een huidcarcinoma van een muis (CA3/7). Uit deze studies kan geconcludeerd worden dat verschillen bestaan tussen de GJIC regulatie in deze twee cellijnen, en dat de korte termijn regulatie van GJIC door intracellulair Ca^{2+}- (Ca^{2+}_i) en door cAMP-afhankelijke processen, twee verschillende routes van regulatie zijn. Een volgende conclusie is dat de aanwezigheid van het cel adhesie molecuul E-cadherin op het plasma-membraan een voorvereiste is voor een hoog GJIC nivo. De hoeveelheid van het E-cadherin eiwit of de plaats van E-cadherin in de cel zijn echter niet betrokken bij de korte termijn-regulatie van GJIC.

De gevonden verschillen in de GJIC regulatie door intracellulaire signaal moleculen tussen 3PC- en CA3/7-cellen suggereren dat gedurende het proces van tumor vorming veranderingen optreden in de Ca^{2+}_i- of calmoduline (CaM)-afhankelijke regulatie van GJIC. Deze verschillen kunnen het gevolg zijn van afwijkende intracellulaire concentraties van de signaal moleculen (zoals Ca^{2+}_i of CaM), of van veranderingen in de gevoeligheid van enzymen die afhankelijk zijn van Ca^{2+}_i- of CaM. Deze verschillen kunnen resulteren in een geblokkeerde intercellulaire communicatie tussen cellen die in een verschillend stadium van tumor vorming verkeren (heterologe communicatie). Indien bijvoorbeeld normale cellen met een hoog GJIC nivo in contact komen met getransformeerde cellen, met een andere Ca^{2+}_i concentratie, dan zal een Ca^{2+} diffusie optreden op het moment dat gap junctions tussen beide cellen functioneel worden. Deze Ca^{2+} diffusie zal leiden tot veranderde Ca^{2+} concentraties in de gap junction regio's in beide cellen, hetgeen kan resulteren in de activatie van mechanismen die GJIC remmen om zo de cellulaire homeostase te bewaren. Dit kan uiteindelijk leiden tot een geblokkeerde heterologe communicatie tussen normale- en getransformeerde cellen, die beide een hoog homoloog GJIC nivo hebben, zoals beschreven voor Balb/c3T3 cellen (9). Het zou daarom interessant zijn om de overdracht van een gap junction permeabele (maar membraan inpermeabele) kleurstof

te bestuderen tussen kleurstof-bevattende cellen en gecocultiveerde getransformeerde cellen, welke normaliter een laag heteroloog GJIC nivo hebben.

Verbindingen met een GJIC-remmende werking (inclusief tumor promotoren) kunnen een rol spelen in de promotie fase van tumor vorming. Verschillende test systemen (gebaseerd op de remming van GJIC) bestaan voor de detectie van deze verbindingen (3,4). In deze systemen worden de doelcellen voor tumor promotoren (geïnitieerde cellen) echter niet gebruikt. Uit het onderzoek beschreven in hoofdstuk 5 kan worden geconcludeerd, dat een cellijn bestaande uit geïnitieerde muis-epidermale cellen (3PC) een goed test systeem is voor de detectie van GJIC-remmende verbindingen, en dat deze cellen gevoeliger zijn voor GJIC-remming vergeleken met een huidcarcimona-afkomstige cellijn. De gevoeligheid van primaire keratinocyten varieerde ten opzichte van 3PC cellen afhankelijk van de gebruikte verbinding.

Om te bepalen of de verschillen in de gevoeligheid voor GJIC remming tussen de celtypen (hoofdstuk 5) te herleiden zijn tot verschillen in mechanismen die mogelijk een rol spelen in de remming van GJIC (connexin hoeveelheid, connexin fosforylatie, connexin locatie in de cel, E-cadherin hoeveelheid en E-cadherin locatie in de cel), bestudeerden we de effecten van tumor promotoren op deze parameters in de verschillende cellen (hoofdstuk 6 en 7). Daar Ca^{2+} een rol speelt in de regulatie van GJIC (hoofdstuk 3), hebben we ook het effect van tumor promoters op Ca^{2+}_i, en de rol van extracellulair Ca^{2+} hierop bestudeerd (hoofdstuk 8). Uit deze studie kan het volgende worden geconcludeerd: 1) de betrokken mechanismen by de remming van GJIC door tumor promotoren zijn afhankelijk van het celtype en van de verbinding; 2) de verschillen in de gevoeligheid voor GJIC-remming tussen de bestudeerde celtypen kan niet geassocieerd worden met effecten van tumor promotoren op een van de bestudeerde parameters; 3) de locatie van E-cadherin in de cellen kan door tumor promotoren worden veranderd; en 4) tumor promotoren kunnen $[Ca^{2+}]_i$ veranderen, maar deze veranderingen zijn niet noodzakelijker wijze verbonden met de remming van GJIC.

De studies met de tumor promotoren in muis epidermale cellen tonen aan dat, in aanvulling op de resultaten van het onderzoek naar de regulatie van GJIC (hoofdstuk 3 en 4), alleen een verminderde immunokleuring van connexin43 (Cx43) op het plasma-membraan gerelateerd kan worden aan een verlaagd GJIC nivo. Er werd geen relatie gevonden tussen andere processen die mogelijk een rol spelen in de regulatie van GJIC (inclusief connexin fosforylatie), en remming van GJIC. Aminozuur analyse van Cx43 kan echter aantonen of de relatie tussen connexin fosforylatie en GJIC

remming überhaupt bestaat.

De mechanismen die het transport van connexins naar- en van het membraan reguleren, alsmede de mechanismen betrokken bij connexon vorming zijn vooralsnog onbekend. Daar verschillende tumor promotoren GJIC remmen in combinatie met een veranderde locatie van zowel Cx43 als E-cadherin, is het interessant om de mechanismen te bestuderen die betrokken zijn bij de inbedding van connexins en E-cadherin in het plasma-membraan. Dit kan leiden tot een verbeterd inzicht in de mechanismen die betrokken zijn bij de (chemisch geïnduceerde) remming van GJIC. Een tijd-gerelateerde studie naar de effecten van tumor promotoren op veranderingen van de Cx43- en E-cadherin immunokleuring kan meer inzicht geven in de rol van E-cadherin in de regulatie van GJIC. Cellen getransfecteerd met een connexin of E-cadherin kunnen mede in dit soort studies nuttige informatie verschaffen.

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Appendix

Curriculum Vitae

Léon Jansen was born in Velp, the Netherlands, on December 4, 1965, where he lived for 1.5 years, after which he moved to Dieren. In 1984 he graduated from the Thomas a Kempis-College in Arnhem and in September he started to study Foodtechnology at the Agricultaral University Wageningen. In August 1990, after 8 months of work at the International Agency for Research on Cancer (IARC), Lyon, France, he graduated for his M.Sc. in Foodtechnology with specialisations in Toxicology, Celbiology and Food Chemistry. After 3 months of work at the Agrotechnological Research Institute (ATO-DLO), Wageningen, he started working as a Ph.D. student for the Department of Toxicology, Agricultural University Wageningen, stationed at the Agrotechnological Research Institute (ATO-DLO). His research project was part of an European project in the program of Science and Technology for Environmental Protection (STEP-project), and was focussed on the effects of tumor promoting agents on gap junctional intercellular communication (GJIC), and the regulation of GJIC during the process of tumor formation, and was under supervision of dr.T.de Vrije, Prof.dr.W.M.F.Jongen, and Prof.dr.J.H.Koeman. Part of this work was carried out at the Department of Multistage Carcinogenesis, IARC, Lyon, France, under supervision of dr.H.Yamasaki and dr.M.Mesnil. Between 1990 and 1994, he also attended the Postgraduate training in Toxicology.

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