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Calcium talk at the blood-brain barrier: *in vitro* studies on calcium signal communication

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De gehele wetenschap is niets meer dan een verfijning van het dagelijks denken.

The whole of science is nothing more than a refinement of everyday thinking.

Albert Einstein

Een woord van dank

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List of abbreviations

α-GA	Alpha-glycyrrhetinic acid
2-NBDG	2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
5-CFDA	5-carboxy-2',7'-dichlorodihydrofluorescein diacetate
A.U.	Arbitrary units
Ac-LDL	Acetylated low density lipoprotein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BBB	Blood-brain barrier
BCEC	Brain capillary endothelial cell
bFGF	Basic fibroblast growth factor
BS	Beam splitter
BSA	Bovine serum albumin
cADPr	Cyclic ADP ribose
CCD	Charge coupled device
CF	6-carboxy fluorescein
CFTR	Cystic fibrosis transmembrane regulator
cGMP	Cyclic guanosine monophosphate
CICR	Calcium-induced calcium release
CNS	Central nervous system
CPS	Counts per second
Cx	Connexin
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DM	Dichroic mirror
EC	Endothelial cell
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FCS	Foetal calf serum
FFA	Flufenamic acid
FITC	Fluorescein isothiocyanate
fluo-3 AM	Fluo-3 acetoxy methylester
FRAP	Fluorescence recovery after photobleaching
GFAP	Glial fibrillary acidic protein
GLUT-1	Glucose transporter type 1
HBSS	Hanks' balanced salt solution

HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	High perfomance liquid chromotography
ICAM	Intercellular adhesion molecule
ICCD	Intensified charge coupled device
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL1-β	Interleukin beta
InsP3	Inositol-1,4,5-trisphosphate
InsP3R	Inositol-1,4,5-trisphosphate receptor
kDa	Kilodalton
LOCC	Ligand operated calcium channel
LY	Lucifer Yellow
MEM	Modified Eagle medium
MS	Mechanical stimulation
MW	Molecular weight
NAADP	Nicotinic acid dinucleotide phosphate
NFA	Niflumic acid
NO	Nitric oxide
NPE	P4(5)-1-(2-nitrophenyl)ethyl
NPPB	5-nitro-2-(3-phenylpropylamino)benzoic acid
PBS	Phosphate buffered solution
PIP	Phosphatidylinositol 4,5-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasmamembrane calcium ATPase
PPADS	Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt
RyR	Ryanodine receptor
SEM	Standard error of the mean
SERCA	Sarcopl-endoplasmic reticulum calcium ATPase
SOCC	Store operated calcium channel
TNF-α	Tumor necrosis factor alpha
TRITC	Tetramethyl rhodamine isothiocyanate
UTP	Uridine triphosphate
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
VOCC	Voltage operated calcium channel
ZO-1	Zonula occludens 1

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Samenvatting

De zogenaamde niet-exciteerbare hersencellen, meer bepaald de elektrisch niet-exciteerbare hersencellen, communiceren via diverse signaalmoleculen daarbij gebruik makend van zowel de intracellulaire als de paracriene signaalweg. De cel-cel communicatie van calciumsignalen is een specifieke vorm van dit algemeen paradigma en specifiek aan deze communicatie is dat deze gekenmerkt is door een alternatieve vorm van prikkelbaarheid, namelijk calcium prikkelbaarheid. Astrocyten reageren op tal van neurotransmitters met een toename van de cytoplasmatische calcium concentratie, wat aantoont dat er een vorm van communicatie bestaat tussen het neuronaal netwerk en de astrocyten. In deze thesis gaan we uit van de vraagstelling hoe calcium signalen gecommuniceerd worden van astrocyten naar de bloedvaten, meer bepaald naar de endotheliale cellen die de bloed-hersen barrière vormen. Calcium signalen worden verondersteld een invloed uit te oefenen op het moleculair transport dat plaatsvindt over de barrière, de laatste stap in de neuron-astrocyte-endotheliale cascade. Onze werkhypothese is dat deze calciumsignalen een functioneel onderdeel zijn van wat wij als “neurobarrière koppeling” willen omschrijven.

In vitro werk met behulp van microscopische calcium beeldvorming en fotoactivatie technieken in astrocyte-endotheliale co-culturen, toonde aan dat calciumsignalen zich voortplanten van astrocyten naar endotheelcellen en omgekeerd. Daarbij wordt gebruik gemaakt van twee fundamentele communicatiewegen namelijk de diffusie van $InsP_3$ doorheen gap junctie kanalen en de vrijstelling van een purinerge boodschapper die diffundeert in het extracellulaire medium en aldus naburige cellen stimuleert. Zowel gap junctie kanalen als purinerge receptoren zijn sterk verbreid in de hersenen, maar de specifieke functie van deze communicatiesystemen ter hoogte van de *in vivo* bloed-hersen barrière dient nader te worden bepaald. Ons verder onderzoek werd gericht naar de communicatie van calciumsignalen tussen de endotheelcellen van de bloed-hersen barrière onderling. Door middel van microscopische calcium beeldvorming, fotoactivatie en bioluminescentie experimenten konden we in endotheliale hersencellen de aanwezigheid van beide fundamentele communicatiewegen aantonen. Bovendien bleek dat deze cellen als antwoord op een toename van de cytoplasmatische $InsP_3$ concentratie, endogeen ATP vrijstellen in het extracellulair medium. Deze gestimuleerde ATP vrijstelling was calcium-afhankelijk en gebeurde via een connexine-gerelateerd mechanisme (gebaseerd op experimenten met gap 26, een connexine mimetisch peptide). Vrijstelling van cellulair ATP kon eveneens veroorzaakt worden door blootstelling van de cellen aan nul extracellulaire calcium condities. Omgekeerd wordt ook de opname van kleurstoffen gestimuleerd ten gevolge van de nul calcium stimulus, wat suggereert dat het mechanisme voor de vrijstelling van ATP in beide richtingen doorlaatbaar is. Uit een vergelijking van de gevoeligheid voor verschillende inhibitoren bleek dat er zowel overeenkomsten

als verschillende bestaan tussen de effecten uitgelokt door de experimentele protocols. Experimenten met stabiele connexine-getransfecteerde HeLa cellen toonden bovendien aan dat inhibitie door connexine mimetische peptiden (gap 26 en gap 27, twee peptiden die een sequentie op respectievelijk de eerste en de tweede extracellulaire lus mimeren) slechts optrad wanneer connexine-43 in de cel aanwezig was. Dit illustreert dat de inhibitie een gevolg is van een specifieke interactie tussen de peptiden en de connexine subunits. Een interessante bevinding was het feit dat de connexine mimetische peptiden en de trivalente ionen gadolinium en lanthanum, ATP vrijstelling blokkeerden zonder dat de intercellulaire koppeling beïnvloed werd. Dit kenmerk maakt van deze substanties interessante instrumenten om de functie van twee verschillende connexine configuraties te onderscheiden: enerzijds de hemikanaal vorm die betrokken is bij de ATP vrijstelling en anderzijds de kanaal vorm in gap juncties.

In verder onderzoek bestudeerden we de relatie tussen endotheliaal calcium en TNF-α, omdat zowel calcium als cytokines zoals TNF-α belangrijke effecten uitoefenen op de permeabiliteit van de bloed-hersen barrière. De bedoeling was te bepalen of TNF-α een invloed heeft op de signaalwegen van cel-cel calcium communicatie. De studie toont aan dat TNF-a zowel de intracellulaire als de paracriene signaalweg inhibeerde, wat suggereert dat substanties die een invloed uitoefenen op de gap juncties dus meestal ook de hemikanaal functie beïnvloeden, analoog aan het effect van substanties zoals α-glycyrrhetinezuur. Een tweede belangrijke conclusie uit deze studie is dat TNF-α de eerste stap van de paracriene signalisatie blokkeert, namelijk de vrijstelling van ATP. Dit betekent dat alle vormen van paracriene ATP communicatie, dus niet alleen de calciumsignaal communicatie, het zwijgen worden opgelegd door het cytokine.

Tot slot, toonden we aan dat zowel de intercellulaire calcium golven als de ATP vrijstelling die optreedt als antwoord op stimulatie met intracellulair $InsP_3$, geen volledige verzadiging vertonen bij $InsP_3$ concentraties in de grootte orde van 10 µM. Deze bevinding duidt op de betrokkenheid van een $InsP_3$ receptor met een lage affiniteit. Een éénmalige stimulatie met 10 µM $InsP_3$ induceerde bovendien de vrijstelling van 1-2 % van de totale ATP inhoud.

Samengevat kunnen we stellen dat een toename van intracellulair $InsP_3$ twee verschillende signaalwegen voor calciumsignaal communicatie induceert: een intracellulaire en een paracriene purinerge signaalweg die optreedt tussen astrocyten en endotheelcellen in co-culturen. De toename aan intracellulair $InsP_3$ en de daaropvolgende calcium toename, veroorzaken de opening van connexine hemikanalen die aanleiding geven tot de vrijstelling van ATP. Bepaalde connexine mimetische peptiden zijn efficiënte blokkers van deze signaalweg, terwijl ze geen effect uitoefenen op de gap junctie kanalen. TNF-α inhibeert deze twee connexine-gerelateerde intercellulaire communicatiewegen.

Summary

So called non-excitable brain cells, i.e. electrically non-excitable brain cells, communicate with each other through gap junctional and paracrine signaling involving diverse signaling molecules. The cell-to-cell communication of calcium signals is a specific form of this general paradigm, but a distinct characteristic of calcium signals is that they provide the cell with a alternative form of excitability namely calcium excitability. Astrocytes respond to neuronally released transmitters such as glutamate with an increase of their cytoplasmic calcium concentration, establishing a communication link between the neuronal network and glial cells. The starting point for this work was the question how astrocytic calcium signals could be further communicated towards the brain vessels, more specifically towards the endothelial cells that form the blood-brain barrier. At this final step of the neuron-astrocyte-endothelial cascade, calcium signals are expected to influence the important molecular transports taking place at the barrier, thereby functioning as a signal involved in what we call “neurobarrier coupling”.

Our *in vitro* work in astrocyte-endothelial co-cultures, applying microscope calcium imaging and photoactivation techniques, indicates that calcium signals are communicated between astrocytes and endothelial cells in a bidirectional way by the diffusion of $InsP_3$ through gap junction channels and by the release of a purinergic messenger diffusing in the extracellular space and acting on neighboring cells. Both gap junctions and purinergic receptors are ubiquitously distributed in the brain, but the precise role of these communication systems in the *in vivo* blood-brain barrier needs to be established. Further work was directed to the communication pathways of calcium signals among endothelial cells. Microscope calcium imaging, photoactivation and bioluminescence experiments in various brain endothelial cell lines illustrated the presence of the two fundamental communication pathways in these cells and, more importantly, demonstrated that these cells release ATP in the extracellular space when triggered with cytoplasmic $InsP_3$ elevation. This triggered ATP release is calcium-dependent and experiments with a connexin mimetic peptide (gap 26), demonstrate a connexin-related mechanism. Cellular ATP release can also be triggered by exposure of the cells to zero extracellular calcium solutions. In addition, uptake of a reporter dye into the cells is also stimulated in response to the zero calcium stimulus, indicating that the ATP release pathway is bidirectionally permeable and thus conductive. Comparison of the sensitivity of the effects evoked by these experimental protocols to various inhibitor substances displayed similarities but also some differences. Work with transfected HeLa cell lines showed that inhibition with connexin mimetic peptides (gap 26 and gap 27, two peptides mimicking a sequence on the first and second extracellular loop of connexins respectively) was only observed when the appropriate connexins were present, illustrating that inhibition is the consequence of specific interactions between the peptides and the connexin subunits. A very interesting observation was

the fact that connexin mimetic peptides, and the trivalent ions gadolinium and lanthanum, block ATP release without influencing gap junctional coupling. This feature converts these substances into interesting tools to dissect the functioning of two distinct configurations of connexins: either the hemichannel form involved in triggered ATP release, or the channel form as present in gap junctions.

Both endothelial calcium and cytokines like TNF-α have important effects on the permeability of the blood-brain barrier, and in further work we investigated cross-talk between these two messengers. The specific aim here was to determine whether TNF-α could influence the two pathways of calcium signal communication. The work showed that TNF-α indeed blocks both the gap junctional and paracrine pathways, demonstrating that substances acting on gap junctions most often also influence connexin hemichannel functioning, just as observed with e.g. α-GA. Another important conclusion from this work is that TNF-α blocked the first step of paracrine signaling, namely the release of ATP. This means that all forms of paracrine ATP signal communication, not solely calcium signal communication, will be silenced by the cytokine.

Finally, we found that the responses to stimulation with intracellular $InsP_3$, either the intercellular calcium wave response or the ATP release response, do not fully saturate in the 10 µM concentration range, indicating the involvement of low affinity receptor sites in these responses. One pulse of ten micromolar $InsP_3$ elevation furthermore triggered 1-2 % of the total cellular ATP content.

In conclusion, our work shows that elevating intracellular $InsP_3$ triggers two pathways of calcium signal communication: a gap junctional and paracrine purinergic pathway acting between astrocytes and endothelial cells in co-culture. The $InsP_3$ increase and its downstream calcium elevation, apparently instigates the opening of connexin hemichannels allowing the outflow of ATP. Certain connexin mimetic peptides are effective blockers of this pathway, while having limited effects on gap junction channels. TNF-α inhibits these two connexin-related pathways of intercellular communication.

Résumé

Les cellules cérébrales non-excitables, plus particulièrement les cellules non-neuronales, communiquent entre-elles en utilisant deux voies de signalisation: une voie qui passe à travers les jonctions communicantes et une voie paracrine utilisant des messagers divers. La communication de signaux calciques entre les cellules est une forme spécifique de ce paradigme général et caractérisé par une forme chimique d'excitabilité: l'excitabilité calcique. Les astrocytes réagissent avec une élévation de leur concentration en calcium cytoplasmique en réponse aux transmetteurs neuronaux libérés, comme par example la glutamate, établissant de cette façon un lien communicatif entre le réseau neuronal et les cellules gliales. Le point de départ de cette thèse était de savoir comment les signaux calciques astrocytiques pourraient être communiqués vers les vaisseaux cérébraux, plus spécifiquement vers les cellules endothéliales qui forment la barrière hémato-encéphalique. A ce point final de la cascade neurone-astrocyte-cellule endothéliale, les signaux calciques sont supposés d'influencer les transports moléculaires importants qui ont lieu à travers la barrière. Ainsi ils fonctionnent comme un signal de communication dans ce que nous proposons d'appeler le ''couplage neurobarrière''.

Les expériences *in vitro* sur des co-cultures astrocytes-cellules endothéliales, en utilisant la microimagerie calcique et des techniques de photoactivation, indiquent que les signaux calciques sont communiqués entre les astrocytes et les cellules endothéliales. Cette communication bidirectionelle utilise la diffusion d'$InsP_3$ à travers des jonctions communicantes et la libération d'un messager purinergique diffusant dans l'extracellulaire et agissant sur les cellules voisines. Les jonctions communicantes ainsi que les récepteurs purinergiques sont distribués en grandes quantités dans le cerveau. Néanmoins le rôle précis de ces systèmes de communication dans la barrière hémato-encephalique *in vivo* doit encore être établi. La suite des expériences est consacrée à la communication des signaux calciques entre les cellules endothéliales. La microimagerie calcique, la photoactivation et les expériences de bioluminescence dans des divers lignées de cellules endothéliales cérébrales démontrent la présence des deux voies de communication de signaux calciques (jonctionelle/paracrine). Plus important, ils démontrent également que ces cellules libèrent de l'ATP dans l'extracellulaire en réponse à une stimulation avec l'$InsP_3$. Cette libération d'ATP est dépendante du calcium cytoplasmique et liée à la présence de connexines (basé sur des expériences avec des peptides mimétiques des connexines). La libération d'ATP cellulaire peut aussi être activée par l'exposition des cellules à une condition extracellulaire de zéro calcium. Aussi l'entrée d'un reporteur fluorescent dans les cellules est stimulée en réponse à un stimulus zéro calcium, ce qui indique que la voie de signalisation de libération d'ATP est perméable de façon bidirectionnelle. La comparaison de la sensitivité de ces protocoles, pour une série d'inhibiteurs de composition chimique diverse, a démontré des

similarités ainsi que des différences. Cette similarité de sensitivité envers les substances inhibitoires a aussi été retrouvée pour l'entrée d'un reporteur fluorescent dans les cellules en réponse à un stimulus zéro calcium. Cette observation indique que la voie de signalisation de libération d'ATP est perméable de façon bidirectionnelle. Les inhibiteurs les plus pertinents de la libération d'ATP stimulée ou de l'entrée de reporteurs fluorescents étaient les peptides mimétiques des connexines (gap 26 et gap 27, deux peptides qui mimiquent une séquence sur le premier et deuxième boucle extracellulaire des connexines), les bloqueurs des jonctions communicantes comme l'acide α-glycyrrhétinique (α-GA) et les ions trivalents. Les expériences avec des lignées HeLa transfectées montraient que l'inhibition avec les peptides mimétiques des connexines était observée seulement quand la connexine-43 était présent, illustrant que cet éffet est la conséquence d'interactions spécifiques entre la peptide et les connexines. Une observation intéressante ést le fait que les peptides mimétiques des connexines, et aussi les ions trivalents, bloquaient la libération d'ATP sans influencer le couplage jonctionnel. Cette caractéristique transforme ces substances à des outils intéressants pour discerner la fonction de deux configurations distinctes des connexines: la forme hemicanale impliqué dans la libération stimulée d'ATP, et la forme canale dans les jonctions communicantes.

Le calcium endothélial et les cytokines comme le TNF-α ont des effets importants sur la perméabilité de la barrière hémato-encephalique. Dans les expériences suivantes on a examiné si le TNF-α avait une influence sur les voies de communication de signaux calciques. Notre travail montre que le TNF-α inhibe la communication jonctionelle et la communication paracrine purinergique. En plus, les substances ayant un effet sur les jonctions communicantes, influencent le plus souvant aussi la fonction des hémicanaux connexines, comme a été observé avec l'α-GA. Une autre conclusion importante est que le TNF-α bloque la première partie de la cascade paracrine, la libération d'ATP. Ça veut dire que toutes les formes de communication paracrine purinergique, non seulement la communication des signaux calciques, seront supprimées par la cytokine.

Finalement, on a trouvé que les réponses à une stimulation avec l'$InsP_3$ intracellulaire, comme la réponse sous forme d'onde calcique ou de libération d'ATP, ne démontre pas de saturation complète dans l'orde de concentrations de dix micromolaires. Cela indique que des récepteurs d'$InsP_3$ d'affinité faibles sont impliqués dans ces réponses. Une seule stimulation avec dix micromolaires d'$InsP_3$ résultait en une libération de 1-2 % de la contenu cellulaire d' ATP.

En conclusion, nos expériences montrent qu'une élévation d'$InsP_3$ engendre deux voies de communication des signaux calciques: une voie de signalisation à travers les jonctions communicantes et une voie de signalisation purinergique paracrine entre les astrocytes et les cellules endotheliales en co-culture. Cette élévation d'$InsP_3$ et l'élévation de calcium consécutive,

semble activer l'ouverture des hémicanaux connexines, permettant la libération d'ATP. Certaines peptides mimétiques des connexines fonctionnent comme inhibiteurs efficaces de cette voie de signalisation, ayant des effets limités sur les jonctions communicantes. TNF-α bloque ces deux voies de communication intercellulaire liées aux connexines.

Chapter

1

Introduction

1.1. Situating the blood brain barrier

In the late 19th century, Ehrlich observed that colored dyes injected into the peripheral circulation of laboratory animals stained all tissues except the brain. Additional experiments by Goldman, a student of Ehrlich, showed that the same dyes injected directly into the cerebrospinal fluid colored the brain but not the peripheral tissues (Goldman, 1913). Clearly, something was preventing these dyes from escaping from the blood vessels in the brain, although they readily leaked from the vessels throughout the rest of the body. These observations brought about the concept of a barrier between blood and brain.

1.1.1. Structure of the BBB

It was nearly half-a-century between the time the blood-brain barrier was discovered and the physical basis of this shield was finally identified. Researchers proved that endothelial cells of the brain microvasculature provide the physical barrier between blood and the central nervous system (Fig. 1.1) (Brightman, 1977; Reese *et al.*, 1967). This is accomplished by the existence of cellular tight junctions and the lack of fenestrations in the endothelial layer. Transport through the barrier is strictly limited, thus allowing for tight control of brain interstitial fluid composition. Two main factors contribute to the BBB's restrictive nature: the physical barrier comprised by the tight junctions between the endothelial cells (Kniesel *et al.*, 2000; Wolburg *et al.*, 2002) and the metabolic barrier comprised by the rich amount of enzymes expressed at the endothelium (Drewes, 2001).

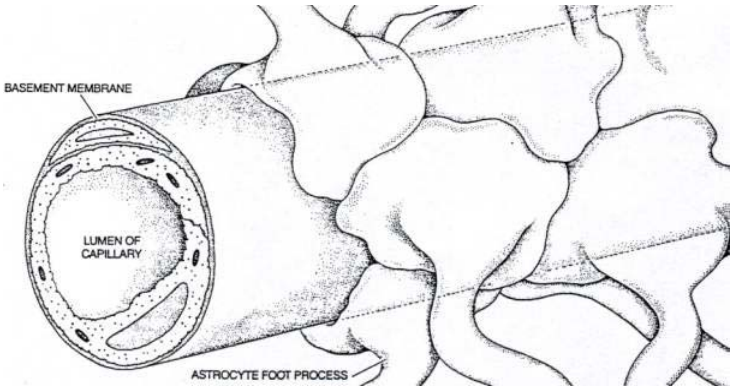

Fig. 1.1 *A schematic representation of the BBB. The capillary endothelium is surrounded by a basement membrane and a sheath of astrocytic processes (end-feet).*

The microcapillaries are ensheathed by outgrowths of astrocytes (Fig. 1.1), which are referred to as astrocytic endfeet (Goldstein *et al.*, 1986). Although astrocytes do not contribute to the physical barrier of the BBB, there is significant evidence indicating that astrocytes are implicated in the maintenance, functional regulation and the repair of the blood brain barrier (Sobue *et al.*, 1999). In addition, complex interactions between other constituents of the microenvironment surrounding the endothelial cells, such as the basement membrane, pericytes, axons, and microglial cells take place and are required for the proper functioning of the blood-brain barrier (Rubin *et al.*, 1999).

1.1.2. Function of the BBB

The most important function of the BBB is to ensure the proper maintenance of the neuronal microenvironment (Pardridge, 1999). This function is accomplished by employing selective transport mechanisms to maintain the proper ion balance within the brain. Even minor changes in the composition or concentration of the ionic solution in the brain can affect nerve transmission.

Another important task of the blood-brain barrier is to help provide neurons with their exact nutritional requirements. Glucose is the sole source of energy for these cells - the adult brain converts about 100 g of glucose to energy each day - and they depend on a steady supply at an exact concentration. Although the concentration of glucose in the blood is controlled within fairly narrow limits by a hormonal system, this regulation is not sufficient for normal brain function. Instead, the BBB transports exactly the correct amount of glucose from the blood into the brain's fluids, which in turn supply it to the cells.

Yet another function of the barrier is to prevent potentially harmful substances from moving out of the bloodstream into the brain or cerebrospinal fluid. The BBB is like a gateway to the brain, keeping out many diseases. Unfortunately, the BBB is often the rate-limiting factor in determining permeation of therapeutic drugs into the brain. To the BBB, drugs are just more foreign material that should be excluded from entering the brain fluids. A better understanding of this selectively impermeable barrier will provide important information that can lead to novel approaches for the treatment of pathologies associated with the central nervous system (CNS), including multiple sclerosis, stroke, meningitis and Alzheimer's disease (reviewed by Mayhan, 2001).

1.2. Important players of the BBB

1.2.1. Endothelial cells

Brain capillary endothelial cells line the inner surface of capillaries in the brain. A unique feature of cerebral endothelium is the degree of organization of the tight junction, which appears as a continuous band surrounding the cells, leaving no room for intercellular clefts (Goldstein *et al.*, 1986). Endothelial cells are characterized by a panel of specific properties, e.g., expression of von Willebrand factor (vWF), a large glycoprotein of complex multimeric structure synthesized by endothelial cells and concentrated in Weibel–Palade bodies (Weibel *et al.*, 1964), uptake of DiI-labeled-acetylated low density lipoprotein (DiI-Ac-LDL) (Robinson *et al.*, 1990) and lectin binding. Endothelial cells possess several metabolic enzymes such as γ-glutamyl-transpeptidase (γ-GTP)

(DeBault *et al.*, 1980) and alkaline phosphatase (Dallaire *et al.*, 1991). Finally, Lechardeur *et al.* (1995a) demonstrated the expression of P-glycoprotein on brain capillary endothelial cells, which appears a specific marker for the BBB.

1.2.1.1 Tight junctions

To perform its barrier function, endothelial cells polarize and form a specialized intercellular junctional complex, which results in a high transendothelial electrical resistance of 1500-2000 ohm cm^{-2}, a unique property of these cells compared with nonbrain vascular endothelium (Crone *et al.*, 1982; Sobue *et al.*, 1999).

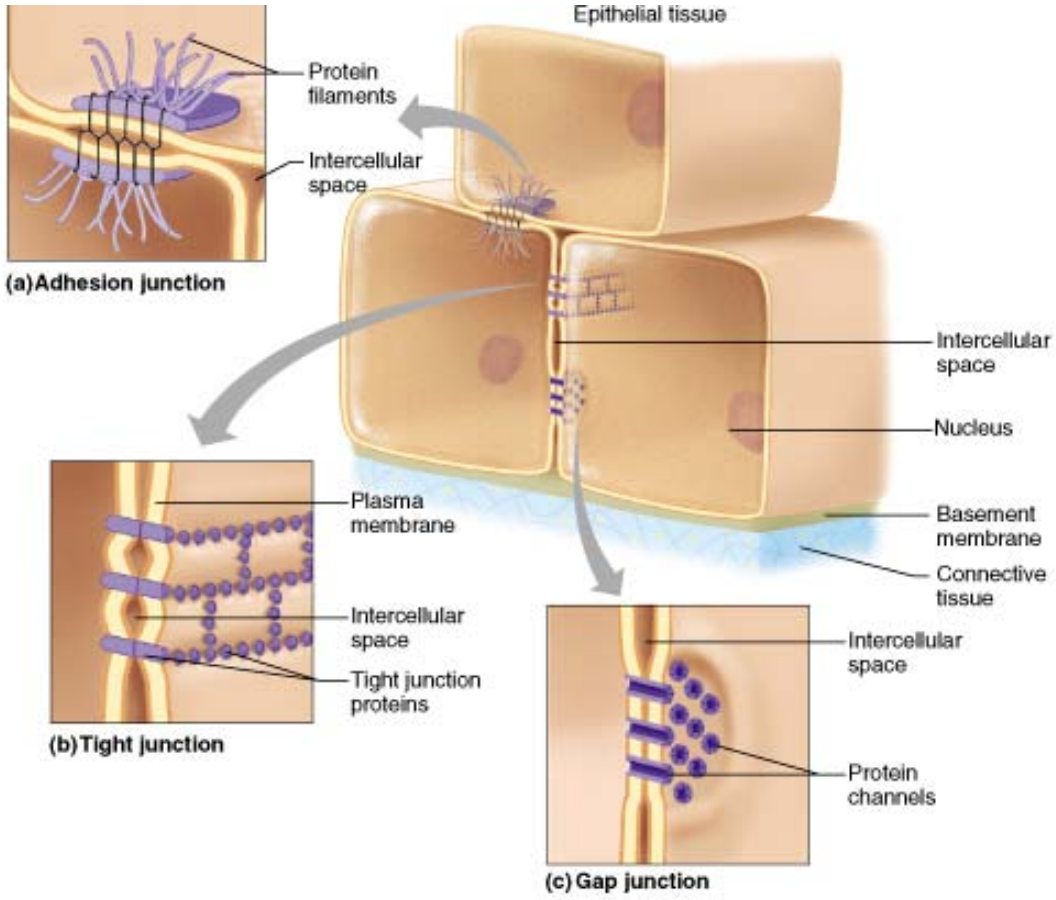

Fig. 1.2 *Specialized junctions occur at the cell-cell contacts between cerebral endothelial cells. (a) Adherens junctions are connecting sites for bundles of actin filaments. (b) Tight junctions are occluding junctions that play a critical part in maintaining the BBB properties. (c) Gap junctions are communicating junctions composed of clusters of channel proteins that allow molecules smaller than about 1000 daltons to pass directly from the inside of one cell to another.*

Tight junctions are regions of the plasma membrane surface between two cells where there is very close association (Fig. 1.2b). The exact molecular composition of tight junctions has been the subject of intensive investigations over the past few years (Tsukita *et al.*, 1999). To date, three integral membrane proteins serve as cell-cell adhesion molecules: claudin, occludin and junctional adhesion molecule (JAM). Claudin is necessary for the formation and maintenance of the tight junctions. Occludin correlates with tightness of the tight junctions. JAM is important in mediating leukocyte adhesion and migration. These transmembrane adhesion molecules are anchored to the actin cytoskeleton via cytoplasmic proteins such as the ZO-proteins. The possible interaction of these proteins in tight junction regulation has recently been reviewed (Kniesel *et al.*, 2000).

Tight junctions are also functionally and physically linked to adherens junctions (Fig. 1.2a), and together they are known as the apical junction complex. Adherens junctions are formed when cadherins on the adjacent cells bind to each other. Similar to tight junctions, they are connected to the actin cytoskeleton by cytoplasmic factors, including catenins and the actin-binding proteins, vinculin and α-actinin.

1.2.1.2 Transport mechanisms

Another aspect in which cerebral endothelial cells differ from endothelial cells in other tissues is a relatively low number of pinocytotic vesicles and a slow rate of fluid-phase endocytosis (Pakulski *et al.*, 1998). As a consequence, the endothelial cells are equipped with membrane transport proteins and receptors located in the luminal and abluminal plasma membranes of the endothelial cell (Risau *et al.*, 1990). Furthermore, they contain much more mitochondria than normal systemic endothelial cells, and this is to supply energy to the multiple energy-dependent transporters in the BBB.

Only nonpolar, highly lipid-soluble compounds can cross the BBB by dissolving in and diffusing across the endothelial cell membranes, whereas the transport of hydrophilic, polar, large, or protein-bound compounds occurs via receptor-mediated transcytosis. There are several transporters for nutrients and endogenous compounds in brain capillary endothelial cells (Pardridge, 1997), including the hexose transport system, amino acid transport system, monocarboxylic transport system, amine transport system. Most of these transporters function in the direction of influx from blood to brain, although, the presence of efflux transporters, such as P-glycoprotein, from brain to blood has also been demonstrated (Lechardeur *et al.*, 1995a).

1.2.1.3 Endothelial cells in vitro

Some groups use primary isolated cells for their studies, accepting time- and cost-effective

preparation steps and common variations in the cell's physiology. Permanent cell lines provide some advantages above primary cells: better uniformity of different cultures and better availability. To avoid the difficulties encountered in primary culture, immortalized rat brain capillary endothelial cell lines, like RBE4 or GP8.3 have been studied intensively (Greenwood *et al.*, 1996; Roux *et al.*, 1994). These cell lines exhibited a nontransformed phenotype and retained some characteristic markers of brain capillary endothelial cells. But in spite of their evident usefulness in calcium imaging studies, they fail to show high electrical resistance (Lechardeur *et al.*, 1995b). Without a tight endothelial cell layer, the passage of hydrophilic substances is not impaired to the degree observed at the BBB *in vivo*. Those cell lines are therefore of very limited use in transendothelial transport studies, but apear very useful for calcium signaling studies.

1.2.2. Astrocytes

Astrocytes compose about 30 % of the volume of the CNS and they outnumber neurons by about ten to one, yet during the 20th century they were neglected by researchers who thought that brain function could be understood in terms of synaptic transmission between neurons. Glial cells were regarded as a passive non-excitable element of the nervous system, the mere glue that held the neuronal system together (Virchow, 1846). As we enter a new century astrocytes are starting to take over some of the spotlight that was once only focused on the brain's neurons.

Astrocytes, together with the oligodendrocytes, belong to the macroglia. Astrocytes are probably the most diverse population of glial cells. One of their hallmarks is the expression of intermediate filament proteins, vimentin and glial fibrillary acidic protein (GFAP). GFAP represents a reliable and widely used marker of astroglia (Bignami *et al.*, 1977).

Astrocytes have dual destructive and protective roles. The close anatomic relationship between brain endothelial cells and the endfeet of astrocytes suggests a role for astrocytes as a source of the differentiation signals. They are thought to produce BBB inducing factors and to maintain the formation of tight junctions between the capillary microvascular endothelial cells (DeBault *et al.*, 1981). They are involved in homeostatic functions such as maintaining extracellular pH and ionic composition. Moreover, they play a critical role in the development and physiology of the CNS, being implicated in many central aspects of neuronal function. They have been assigned to supply neurons with energy and substrates for neurotransmitter synthesis (Tsacopoulos *et al.*, 1996) and assist with synaptic connectivity regulation (Bacci *et al.*, 1999). Besides these protective features, astrocytes also remove cellular debris (al-Ali *et al.*, 1996).

1.2.3. Models of BBB

The importance of the blood-brain barrier for the maintenance of a constant inner milieu of the brain, is responsible for the great efforts in the establishment of an *in vitro* model. Such a model may provide a useful tool for a better understanding of the blood-brain barrier *in vivo* and for pharmacological studies (Joo, 1993a).

The simplest model of an astrocyte-induced BBB is one using astrocyte-conditioned medium. Another model is a mixed co-culture of endothelial cells and astrocytes, where the two cell types are in direct contact with each other. When the two cell types are grown together, a characteristic morphologic organization occurs that is associated with induction of enzymes and tight junctions similar to those found *in vivo* (Cecchelli *et al.*, 1999; Dehouck *et al.*, 1992). This model therefore represents a useful tool *in vitro* for examination of the cellular physiology, pharmacology and biochemistry of endothelial cells and astrocytes at the BBB. Finally, pial microvessels have been used commonly in studies of the blood-brain barrier because of their relative accessibility (Allt *et al.*, 1997). Although pial microvessel lack the ensheathment of astrocytes characteristic of cerebral microvessels (Goldstein *et al.*, 1986), these vessels have many morphological features of the BBB in common. Nevertheless, these two vessel types differ in other respects, so caution is urged regarding the use of pial microvessels to investigate the blood-brain barrier (Lawrenson *et al.*, 1997).

1.3. Calcium in the BBB

Calcium is a universal signal transduction element in all cells of the CNS where it performs a special role in the maintenance of the basic functional properties. But, the way to an understanding of the mechanism of this crucial role of calcium ions has been long and complicated. Now we know that practically all intracellular messenger systems involve intracellular calcium in their function.

1.3.1. Intracellular calcium signaling

1.3.1.1 Generation of a calcium signal

There are various mechanisms involved in the generation of the cellular calcium signal. Cytosolic calcium is derived from two sources, intracellular stores and the extracellular medium. The basis of calcium signal generation is the existence of a large electrochemical gradient across the

membrane. Outside the cell and in the stores, the free calcium concentration is around 1mM, whereas the free calcium concentration in the cytoplasm of cells is maintained at around 0.1µM (Fig. 1.3).

A major source for the generation of cellular calcium signals in electrically excitable cells is calcium influx across the plasmamembrane. The best known mediator of these calcium signals is the voltage-operated family of calcium channels (VOCC) which are activated by depolarization of the membrane (Hofmann *et al.*, 1994). External calcium also enters the cytoplasm through ligand-operated calcium channels (LOCC) which open upon binding of a specific agonist (Burnashev, 1998). The third family of plasmamembrane calcium channels is represented by store-operated channels (SOCC) (Lewis, 1999) which require the depletion of internal stores as activation signal. Finally, mechanosensitive nonselective cation channels also provide a pathway for calcium influx in endothelial cells, which express various functional mechanosensitive ion channels (Lansman *et al.*, 1987; Nilius *et al.*, 2001; Yao *et al.*, 2000). An inital calcium influx through these channels is followed by calcium release from intracellular calcium stores (Himpens *et al.*, 1999).

An alternative route for calcium signal generation, is calcium release from specialized intracellular calcium stores. Currently, the main intracellular store is the endoplasmic reticulum calcium pool. In the nervous system two different receptor subtypes govern the intracellular calcium stores: the inositol-1,4,5-trisphophate receptor ($InsP_3R$) and the ryanodine receptor (RyR). Many plasma membrane receptors are linked to the phosphatidyl inositide signaling system which involves G-protein mediated activation of phospholipase C that generates $InsP_3$ and diacylglycerol (DAG). $InsP_3$ is known to bind to and activate receptors present on intracellular calcium stores allowing calcium to be released into the cytosol (Berridge, 1993; Bezprozvanny *et al.*, 1995). Cyclic ADP ribose was characterized as the physiological activator of RyR (Galione, 1994). Opening of the $InsP_3R$, in the presence of $InsP_3$, and of the RyR is also stimulated by calcium binding (calcium-induced calcium release, CICR) (Verkhratsky *et al.*, 1996b).

The depletion of calcium stores due to the activation of $InsP_3$ or other calcium releasing signals activates calcium influx across the plasma membrane through a process known as “capacitative calcium entry” or “store-operated calcium entry”. Several models have been proposed for the signaling pathway leading to the opening of the store-operated channels (reviewed by Putney, 2003). There is evidence that depletion of the calcium stores causes a conformational change in the $InsP_3$ receptor, which could be conveyed to the plasma membrane via the cytoskeleton or via more direct protein-protein interactions. This mechanism is called the conformational coupling model (Kiselyov *et al.*, 1998). Alternatively, in a secretion-like coupling model, preformed and active calcium channels are inserted into the membrane by vesicle fusion (Patterson *et al.*, 1999). Third, store-operated calcium channels are kept in an inhibited state by an inhibitory mechanism

when the calcium stores are full, and discharge of calcium stores removes such an inhibitory mechanism (Putney *et al.*, 2001). Finally, it has been suggested that depletion of the calcium stores results in the production of a diffusible messenger, the calcium influx factor (CIF), which affects the gating of membrane channels (Randriamampita *et al.*, 1993; Csutora *et al.*, 1999). However, the chemical nature of CIFs has not been resolved.

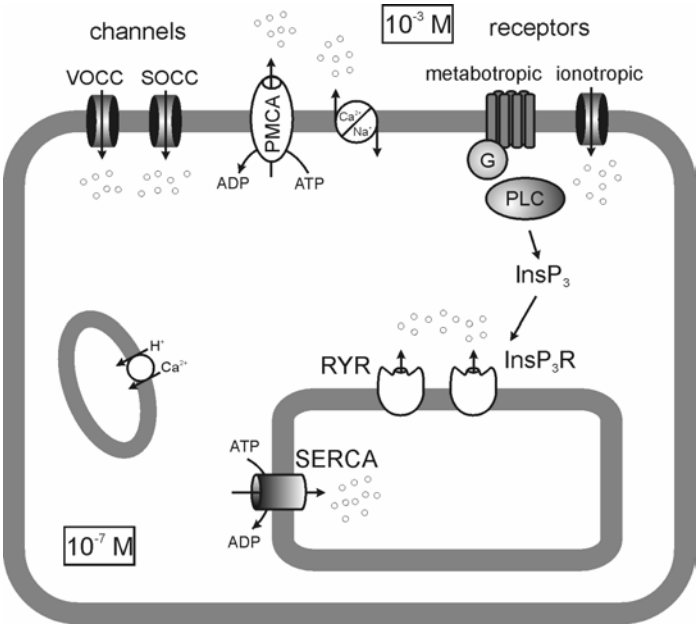

Fig. 1.3 *Principal mechanisms of intracellular calcium signaling in a single cell. Extracellular calcium enters the cell through plasma membrane calcium channels and leaves the cell using calcium pumps and Na^+/Ca^{2+} exchangers. Endoplasmic reticulum (ER) is a major site for sequestered calcium ions. Calcium is accumulated in intracellular stores by means of calcium pumps and released via $InsP_3$ receptors ($InsP_3R$) or ryanodine receptors (RyR).*

1.3.1.2 Calcium buffering and clearance

Unlike many other second messenger molecules, calcium cannot be metabolized. Yet prolonged high intracellular calcium levels lead to cell death, which makes a tight regulation of the calcium concentration inevitable. Cells have therefore evolved complex homeostatic mechanisms which consist of a delicate interplay between influx, efflux, storage and buffering of calcium ions (Ghosh *et al.*, 1995).

One strategy to regulate the cytosolic calcium concentration is to cluster calcium influx channels near the desired site of action. This allows to generate high localized calcium gradients in subcellular areas to trigger only those processes which are located at the site of influx. For example, VOCC in synaptic terminals are co-localized near the sites of calcium-dependent neurotransmitter release (Llinas *et al.*, 1992).

The large concentration gradient from outside to inside in addition to the electrical gradient forces the cell to expend metabolic energy to extrude calcium from the cytosol. The most prominent mechanism which carries calcium across the plasma membrane is the plasma membrane calcium ATPase (PMCA) (Carafoli *et al.*, 1994). In addition, a Na^+/Ca^{2+} exchange transport mechanism

expels calcium ions out of the cell as sodium ions are brought in (Philipson, 1996).

Large quantities of calcium ions can also be sequestered into organelles, including the internal calcium stores and mitochondria. In order to achieve this, they use active and passive calcium transport mechanisms such as the ATP-dependent sarco-endoplasmic reticulum calcium ATPase (SERCA) (Missiaen *et al.*, 1991) and the H^+/Ca^{2+} uniporter in the mitochondria, which is driven by the mitochondrial membrane potential established by the electron transport chain (Thayer *et al.*, 1990).

Cytoplasmic calcium buffering is carried out by a number of intracellular calcium-binding proteins, belonging to the EF-hand family such as calmodulin, calbindin and parvalbumin (Heizmann *et al.*, 1991). These binding proteins have an enormous capacity to buffer calcium. Collectively, they adsorb nearly 95-99 % of the total cellular calcium load, leaving the rest available as free calcium.

1.3.1.3 Appearance of a calcium signal

In both excitable and nonexcitable cells, calcium signals have been suggested to result from spatially and temporally coordinated recruitment of subcellular calcium release units (Bootman *et al.*, 1995; Lipp *et al.*, 1997; Niggli, 1999). Elementary calcium release events (Fig. 1.4) may involve individual $InsP_3Rs$ (calcium blips) and RyRs (calcium quarks) or concerted activation of several receptors within a single release site (calcium puffs and calcium sparks respectively).

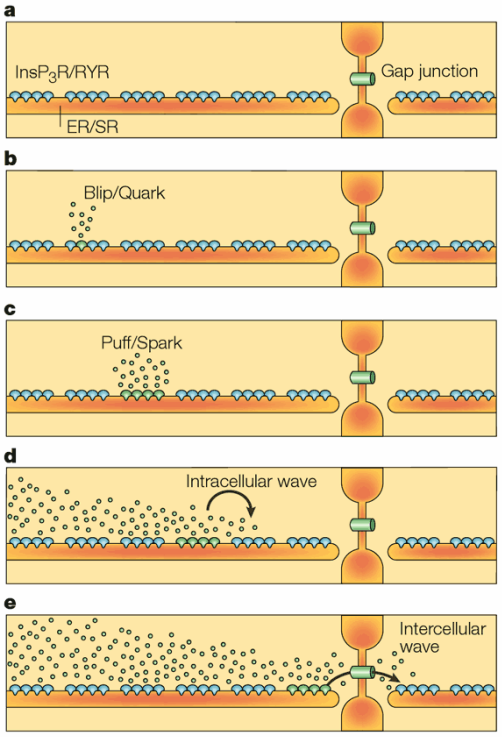

Fig. 1.4 *The spatial organization of calcium release from internal stores. a) $InsP_3$ receptors ($InsP_3Rs$) and ryanodine receptors (RYRs) are distributed over the surface of the endoplasmic and/or sarcoplasmic reticulum (ER/SR). b) In response to weak stimuli, individual channels open to give either blips ($InsP_3Rs$) or quarks (RYRs). c) At higher levels of stimulation, groups of $InsP_3Rs$ or RYRs open together to produce puffs or sparks, respectively. d) When cells are fully excitable, the elementary events depicted in (c) can excite neighboring receptors through a process of calcium-induced calcium release to set up an intracellular wave. e) When gap junctions connect cells, waves can travel from one cell to the next to set up an intercellular wave. Adapted from Berridge et al. (2000).*

Intracellular calcium changes during cellular activity are not only represented by simple elementary events, but also by more complex periodic events (Jaffe, 1991; Thomas *et al.*, 1991). Elementary events form the building blocks underlying the complex spatio-temporal patterns of cellular calcium signals. A major signal mechanism consists of periodic increases of the cytosolic calcium concentration, the so called calcium oscillations. In addition, these oscillations are often spatially organized so that a single intracellular wave originates from one specific region (Berridge, 1997).

The complexity of calcium signals contains a wealth of information encoded in the amplitude and frequency of the calcium signals. Cells use various intracellular calcium-binding proteins to decipher the information and convert the calcium signals into a wide variety of cellular processes. This has recently been illustrated for neuronal differentiation (Gu *et al.*, 1995) and fluid secretion by salivary glands (Rapp *et al.*, 1977). It has also been reported that the amplitude and duration of the calcium signal optimizes gene expression in astrocytes (Li *et al.*, 1998) or Jurkat T-cells (Dolmetsch *et al.*, 1998).

1.3.2. Intercellular calcium signals in the brain

Increases in the intracellular calcium concentration that spread from cell to cell provide a mechanism for cells to coordinate many activities. This phenomenon is called an intercellular calcium wave. These intercellular calcium waves have been studied in many cell types, including neurons (Charles *et al.*, 1996) and glial cells (Charles *et al.*, 1991). There are currently two mechanisms for intercellular calcium wave propagation. One model involves the diffusion of $InsP_3$ through gap junctions (Sanderson *et al.*, 1990) while in the second model, calcium wave are mediated by paracrine activity of released ATP (Osipchuk *et al.*, 1992). In the following review, intercellular calcium signals at the CNS are highlighted.

Calcium signal communication systems in the central nervous system

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1.4. Intercellular calcium signals

Traditionally, neuronal electrochemical impulses are seen as the basis of information processing in the central nervous system (CNS). There is, however, growing evidence that so called “non-excitable” cell types such as glial cells and also brain vessel cells, are actively participating in brain functioning by responding to synaptic activity (Dani *et al.*, 1992), by modulating these information circuits (Kang *et al.*, 1998; Nedergaard, 1994) and by exchanging signals to coordinate the functioning of the basic triad consisting of neurons, glial cells and microvascular cells. Intracellular calcium ions play a pivotal role in non-excitable cells as a messenger for *intra*cellular and *inter*cellular signaling (Berridge *et al.*, 2000; Rottingen *et al.*, 2000; Sanderson *et al.*, 1994). Because of a certain degree of homology between action potentials and calcium transients these signals provide non-excitable cells with some form of ‘calcium excitability’ (van den Pol *et al.*, 1992). Intercellular calcium signals are transient changes in cytoplasmic free calcium that are, analogous to action potentials in neurons, characterized by an initiating trigger followed by a mechanism that propagates the calcium signal to neighboring cells. The velocity of signal propagation is in the order of tens of micrometers per second, i.e. two to three orders of magnitude slower as action potential propagation. The spectrum of intercellular calcium signals ranges from the most elemental form of calcium signal exchange between just a pair of cells up to the massive intercellular calcium waves encompassing hundreds of cells. In its elemental form the calcium signal is propagated by diffusion of a messenger, analoguous to electrotonic (subthreshold) signal spread. In principle, the term calcium wave is to be reserved for calcium signal propagation that involves a regenerative aspect, analoguous to action potential signal propagation. This distinction is however not generally made in the literature and the term calcium wave has been frequently applied to denote calcium signals being communicated over a large population of cells regardless the involvement of regenerative phenomena.

The communication of calcium signals between brain cells is a typical feature of glial cells. It is however not restricted to this kind of cells as neurons, also exchange calcium signals among each other (Charles *et al.*, 1996; Yuste *et al.*, 1995) as well as with the surrounding astrocytes (Charles, 1994; Dani *et al.*, 1992). Extensive research over the past ten years has identified several calcium signal communication systems in the brain and has in addition put forward possible roles or functions. In this paper we review the data available on cell-to-cell communicated calcium signals in the central nervous system. We focus on the various pathways involved in the cell-to-cell propagation of calcium signals and highlight the role of these signals in communication systems such as neuroglial and gliovascular signaling.

1.4.1. Calcium signal communication mechanisms

Two mechanisms have been identified that support cell-to-cell communication of calcium signals in various cell types. The first mechanism involves the diffusion of a calcium mobilizing messenger, or calcium itself, through gap junction channels (Sneyd *et al.*, 1995). The second mechanism relies on paracrine signaling (Hassinger *et al.*, 1996; Osipchuk *et al.*, 1992) involving the release of a messenger, diffusion in the extracellular space, binding to receptors on neighboring cells and activation of downstream signaling cascades that ultimately lead to an increase of cytoplasmic free calcium in the target cell. The calcium increase in the target cell might on its turn initiate a new cycle of calcium signal communication, i.e. cause re-generation of the cell-cell calcium signaling event producing regenerative calcium signal propagation (Domenighetti *et al.*, 1998) thereby spreading the signal over a larger population of cells as compared to the purely diffusive spread of calcium or calcium mobilizing messengers between cells.

1.4.1.1 Gap junctional calcium signal communication

Gap junctions are the sites of direct cell-to-cell communication, facilitating the exchange of chemical and electrical signals between cells. These intercellular channels are composed of two hexameric half-channels, called connexons or hemichannels (Goodenough *et al.*, 1996). The end-to-end interaction or “docking” of two connexons, each provided by one of the two neighboring cells, generates an intercellular aqueous channel that allows the exchange of nutrients, metabolites, ions and small molecules with a molecular weight up to ~1000 Dalton (single channel conductance in the order of 100 pS). Gap junctions are ubiquitous throughout the central nervous system (Dermietzel *et al.*, 1993; Rouach *et al.*, 2002; Spray *et al.*, 1996) and are covered by several recent reviews describing their structure and function (Duffy *et al.*, 2002; Evans *et al.*, 2002; Goodenough *et al.*, 1996; Shibata *et al.*, 2001).

The importance of gap junction channels in communicating calcium signals between cells has become clear since the direct demonstration that calcium itself and the calcium mobilizing messenger inositol trisphosphate ($InsP_3$) can permeate through gap junction channels (Saez *et al.*, 1989). Subsequent work by others has extensively demonstrated the functionality of $InsP_3$ diffusion through gap junctions as a calcium signal propagating mechanism (Boitano *et al.*, 1992; Giaume *et al.*, 1998; Leybaert *et al.*, 1998; Sanderson *et al.*, 1994; Sanderson *et al.*, 1990). Further work has also demonstrated that the permeability of gap junction channels to $InsP_3$ depends on the connexin subtype being involved (Niessen *et al.*, 2000). Besides $InsP_3$ and calcium, the calcium mobilizing messenger cADP ribose has also been proven to be a trigger of intercellular calcium signals but its contribution to the intercellular propagation of the calcium signal seems to be dependent on the

$InsP_3$-generating activity of PLC (Churchill *et al.*, 1998; Leybaert *et al.*, 2001). Although calcium ions themselves can diffuse through gap junction channels, their role in communicating calcium signals between cells is limited because their diffusion is severely hindered by binding to more slowly diffusing cytoplasmic calcium buffers, which makes the effective diffusion in the cytosol constant much lower as compared to that of $InsP_3$ (D_{eff} Ca^{2+} = 20 $\mu m^2 s^{-1}$; D_{eff} $InsP_3$ = 300 $\mu m^2 s^{-1}$; Allbritton *et al.*, 1992; Sneyd *et al.*, 1995). Under certain conditions however, calcium can act as an effective calcium signal messenger between cells (Hofer *et al.*, 2001). One such condition is the priming of $InsP_3$ receptors by exposure to slightly elevated background $InsP_3$ concentrations, which brings these receptors in a more calcium-sensitive state (Kaftan *et al.*, 1997). Under these conditions, calcium-induced calcium release, taking place at both $InsP_3$ and ryanodine receptors, brings the cytoplasm into a calcium-excitable state and thus susceptible to stimulation by calcium diffusing in from adjacent cells through gap junction channels (Yule *et al.*, 1996). An important aspect of calcium signal communication by diffusion of $InsP_3$ through gap junction channels is that even small amounts of $InsP_3$ flowing into a cell, e.g. because of a very low level of gap junctional coupling, are sufficient to produce measurable calcium signals because of the amplification effect associated with calcium-induced calcium release (Berridge, 2002; Braet *et al.*, 2001).

1.4.1.2 Paracrine calcium signal communication

Osipchuk *et al.* (1992) provided the first evidence that an extracellular messenger mediates the spreading of cell-to-cell calcium signals in mast cells. In principle, every substance that can be released by a cell and can cause a rise in cytoplasmic calcium in neighboring cells, is a calcium messenger candidate. However, not all possible candidate messengers (reviewed for glial cells by Gimpl *et al.*, 1993; Kastritsis *et al.*, 1993; Kirischuk *et al.*, 1996; Verkhratsky *et al.*, 1996a; Verkhratsky *et al.*, 1998) have proven to effectively communicate calcium signals between brain cells. Most evidence is presently available for substances like ATP, glutamate and nitric oxide (NO) (Guthrie *et al.*, 1999; Hassinger *et al.*, 1996; Leybaert *et al.*, 1998; Malcolm *et al.*, 1996; Parpura *et al.*, 1994; Willmott *et al.*, 2000a). The exact type of messenger involved depends on the cell type and paracrine calcium signal communication will therefore be more specifically dealt with in the next chapter where calcium signal communication systems are considered.

1.4.2. Calcium signal communication systems

Calcium signal communication is a feature of both neurons and glial cells. Neurons mainly communicate electrical signals between each other, but the exchange of calcium signals is also operative (Charles *et al.*, 1996; Yuste *et al.*, 1995) and is intimately related to synaptic functioning. An increase of calcium by calcium entry through P- and Q-type calcium channels and to a lesser

extent through N- and R-type calcium channels is the triggering step of exocytosis in the presynaptic terminal (Meir *et al.*, 1999; Qian *et al.*, 2001; Wu *et al.*, 1999). Postsynaptic calcium changes are induced at the level of dendritic spines by glutamate acting on NMDA-, AMPA- and metabotropic receptors, by voltage-sensitive calcium entry and by release from various dendritic calcium stores (Connor *et al.*, 1994; Segal, 1995). Dendritic spines behave like calcium microdomains connected to the dendritic tree, and intraspine calcium signals regulate synaptic efficacy and plasticity, as demonstrated in phenomena like long-term potentiation and long-term depression (Blackstone *et al.*, 2002; Takechi *et al.*, 1998), two cellular mechanisms implicated in memory and learning. The field of neuronal communication of calcium signals is immense and will not be dealt with in this review.

Changes in cytoplasmic calcium concentration form the predominant signal by which glial cells regulate their own activity and influence neuronal behavior (Scemes, 2000). Calcium signals can be communicated among astrocytes, but also between astrocytes and multiple other cell types, including neurons (Araque *et al.*, 1998b; Kang *et al.*, 1998; Parri *et al.*, 2001), microglia (Schipke *et al.*, 2002) and vascular endothelial cells (Leybaert *et al.*, 1998; Paemeleire *et al.*, 2000a), often appearing as a two-way signal (Fig. 1.5, Table 1.1). Several recent reviews discuss glial calcium signaling in full detail (Kettenmann *et al.*, 2003; Scemes, 2000) and we narrow the focus of the present review only to communication aspects of calcium signals.

Table 1.1 Calcium signal communication systems

system	propagating signal	references
neuron-astrocyte	gap junctions	Nedergaard (1994)
	glutamate	Porter *et al.* (1996); Pasti *et al.* (1997)
	NO	Matyash *et al.* (2001)
	acetylcholine	Araque *et al.* (2002)
astrocyte-neuron	gap junctions	Nedergaard (1994)
	glutamate	Parpura *et al.* (1994); Hassinger *et al.* (1995); Bezzi *et al.* (1998)
astrocyte-astrocyte	gap junctions	Charles *et al.* (1993)
	ATP	Guthrie *et al.* (1999); Cotrina *et al.* (2000); Wang *et al.* (2000)
	glutamate	Innocenti *et al.* (2000)
	NO	Willmott *et al.* (2000a)
astrocyte-microglia	ATP	Verderio *et al.* (2001); Schipke *et al.* (2002)
astrocyte-endothelial cell	ATP and gap junctions	Leybaert *et al.* (1998); Paemeleire *et al.* (2000b) Braet *et al.* (2001)
astrocyte-meningeal cell	ATP and gap junctions	Grafstein *et al.* (2000)
endothelial cell-endothelial cell	ATP and gap junctions	Braet *et al.* (2003b)

1.4.2.1 Calcium signals between astrocytes

Intercellular calcium waves have been described between astrocytes in culture and in organotypic and acute brain slices (Charles *et al.*, 1991; Cornell-Bell *et al.*, 1991; Finkbeiner, 1992). Astrocytic calcium waves appear to propagate either via the intracellular gap junctional pathway (Sanderson *et al.*, 1994; Venance *et al.*, 1997) or via the extracellular paracrine pathway (Charles, 1998; Guthrie *et al.*, 1999; Hassinger *et al.*, 1996). One of the major arguments for the role of gap junctions in calcium signal communication between these cells has been based on comparing calcium signal propagation in connexin transfected and wild type glial cell lines (Charles *et al.*, 1992). However, subsequent work from Cotrina *et al.* (1998a) has demonstrated that connexin transfection is also associated with an increased release of paracrine acting messengers like ATP, thereby fundamentally challenging the interpretation of this kind of experiments. Work with antisense oligonucleotides and with connexin knock-out animals seems to confirm this observation and furthermore demonstrates that changes in connexin expression induces shifts in purinergic receptor subtype expression (Scemes *et al.*, 2000; Suadicani *et al.*, 2003). Much of the interest in the mechanism of calcium signal communication between cells has since then shifted towards investigations on the paracrine pathway.

Astrocytes express a myriad of different neurotransmitter receptors (Porter *et al.*, 1995) opening up a large list of candidate extracellular messenger substances. The key extracellular mediator in paracrine calcium signal communication has been identified as ATP (Guthrie *et al.*, 1999). The mechanisms by which astrocytes release ATP appear to be diverse (Table 1.2). There is evidence for vesicular release (Bal-Price *et al.*, 2002; Coco *et al.*, 2003; Volknandt, 2002) as well as for release through connexin hemichannels, which are half gap junction channels not connecting to neighboring cells (Cotrina *et al.*, 1998a; Stout *et al.*, 2002). This last mechanism has quickly brought the focus of interest back to the connexins, opening up the possibility for their involvement in both gap junctional and paracrine calcium signal communication (Goodenough *et al.*, 2003). A major problem concerns the interpretation of work performed with gap junction blockers, because these affect both gap junction channels and connexin hemichannels making it difficult to decide which of both is involved. A promising approach to dissect the two pathways is offered by the use of connexin mimetic peptides, which -when applied over a short time period- only affect connexin hemichannels without influencing the gap junction channels (Braet *et al.*, 2003b; Braet *et al.*, 2003c; Leybaert *et al.*, 2003). Other astrocytic ATP release pathways include the ATP-binding-cassette transporters such as the cystic fibrosis transmembrane regulator (CFTR) or the P-glycoprotein (Ballerini *et al.*, 2002; Darby *et al.*, 2003; Queiroz *et al.*, 1999). The relative contribution of each of these proposed release pathways is currently not known and it is equally unclear whether multiple pathways work perhaps together to form a complex cascade of ATP

release. Conductive ATP release pathways like connexin hemichannels will indeed be expected to allow, in addition to passing ATP, calcium to enter the cells thereby perhaps activating other release pathways, and autocrine ATP actions might further add to make the sequence of events even more complex.

Table 1.2 Different mechanisms of ATP release

cell type	stimulus	proposed mechanism	references
astrocytes	hypotonicity	P-glycoprotein	Darby *et al.* (2003)
		ABC-transporters	Ballerini *et al.* (2002)
	mechanical stimulation	hemichannels	Stout *et al.* (2002)
		vesicular	Coco *et al.* (2003)
	UTP	hemichannels	Cotrina *et al.* (1998a)
	glutamate	CFTR or CFTR-related	Queiroz *et al.* (1999)
	low calcium	hemichannels	Arcuino *et al.* (2002)
endothelial cells	$InsP_3$	hemichannels	Braet *et al.* (2003b)
	low calcium	hemichannels	Braet *et al.* (2003b)
	mechanical stimulation	vesicular	Bodin *et al.* (2001a)

In addition to ATP, ADP might also be involved as a paracrine messenger: ADP is formed by enzymatic (ectonucleotidase) degradation of ATP (Zimmermann *et al.*, 1998) and might in principle also be released by the cells through conductive pathways. Experimental evidence for ADP as a paracrine acting calcium mobilizing messenger is however only available from work in non-glial cells (Moerenhout *et al.*, 2001). UTP is another paracrine messenger candidate in glial cells (Harden *et al.*, 1999; Lazarowski *et al.*, 1997). ATP, ADP and UTP all trigger calcium changes in astrocytes and subsequent increases in calcium can trigger ATP release (Cotrina *et al.*, 1998b; Queiroz *et al.*, 1999). ATP release is, however, not always calcium-dependent and varies with the trigger or the release pathway being involved. NO-induced vesicular ATP release (Bal-Price *et al.*, 2002) and low calcium-induced connexin hemichannel related ATP release are reported to be calcium-dependent (Arcuino *et al.*, 2002). NMDA-induced ATP release is dependent on calcium entry whereas AMPA-induced ATP release does not require calcium influx (Queiroz *et al.*, 1999). In addition, calcium-independent ATP release was reported by Wang *et al.* (2000). Calcium-dependent release will be expected to mediate far reaching regenerative calcium signal propagation while the calcium-independent forms are expected to operate short range calcium signal communication.

Adenine and uridine nucleotides act as agonists to increase cytoplasmic calcium in astrocytes (Khakh *et al.*, 2001; King *et al.*, 2000). Purinergic receptors are divided into ionotropic P_2X receptors and metabotropic P_2Y receptors (reviewed by Ralevic *et al.*, 1998). P_2Y receptors are G-protein linked receptors that increase intracellular $InsP_3$ and mobilize calcium from $InsP_3$-sensitive stores while P_2X receptors are ligand-gated cation channels that mediate calcium entry into the cell. P_2Y receptors are activated by nanomolar ATP concentrations while micromolar concentrations are needed to activate P_2X receptors (James *et al.*, 2001). Primary rat cortical

astrocytes express all cloned metabotropic P_2Y receptors that are activated by both adenine and uridine nucleotides with different potency rank orders, but only some are functionally coupled to calcium increases (P_2Y_1, P_2Y_2, P_2Y_4, and P_2Y_{14}) (Fumagalli *et al.*, 2003; Zhu *et al.*, 2001). The involvement of purinergic receptors in calcium signal communication has been demonstrated by applying broad spectrum purinergic receptor antagonists (Cotrina *et al.*, 1998b; Guthrie *et al.*, 1999; John *et al.*, 1999a). Further work with subtype-selective inhibitors or with selective expression of the receptor subtypes has clearly demonstrated the involvement of P_2Y_1 and P_2Y_2 receptors in calcium signal propagation in spinal cord astrocytes and ha elucidated their role in conferring distinct charateristics to the calcium wave depending on the receptor subtype involved (Fam *et al.*, 2003; Gallagher *et al.*, 2003; Koizumi *et al.*, 2002; Scemes *et al.*, 2000). Astrocytes in primary culture also express various subtypes of ionotropic P_2X receptors (P_2X_2, P_2X_4, P_2X_5 and P_2X_7) of which at least P_2X_7 has directly been demonstrated to mediate ATP-induced calcium rises (Fumagalli *et al.*, 2003).

Studies by Willmott *et al.* (2000a) have demonstrated that NO is able to trigger intercellular calcium waves and is also involved in the cell-to-cell propagation of the calcium signal in mixed glial-neuronal cultures, which contain a majority of astrocytes. NO is synthesized by enzymatic oxidation of L-arginine by nitric oxide synthase (NOS) (Lowenstein *et al.*, 1992) that exists in three isoforms in the CNS: neuronal NOS (nNOS), endothelial NOS (eNOS) also present in astrocytes and microglia (Murphy *et al.*, 1993a; Shafer *et al.*, 1998) and inducible NOS (iNOS) in endothelial cells, microglia, astrocytes and neurons (Nathan *et al.*, 1994). nNOS and eNOS are constitutively expressed and synthetize NO in response to increases in cytoplasmic calcium levels. NO activates guanylyl cyclase and increases cytoplasmic cGMP which, via a cGMP-dependent kinase, activates ADP-ribosylcyclase to produce cADP ribose (Galione *et al.*, 1993). In astrocytes, cADP ribose triggers ryanodine receptor mediated calcium release (Willmott *et al.*, 2000a), but NO also appears to increase calcium through cGMP-independent mechanisms, either by mobilizing calcium from stores (Bal-Price *et al.*, 2002; Bowman *et al.*, 2001) or by stimulating calcium entry (Willmott *et al.*, 2000b). The relative contribution of ATP and NO as paracrine messengers of calcium signals between astrocytes is currently unknown.

Next to purinergic and nitrergic messengers, astrocytic calcium waves have also been demonstrated to be associated with a spatially spreading increase in extracellular glutamate (Innocenti *et al.*, 2000). Astrocytic glutamate release is either vesicular (Araque *et al.*, 2000; Bal-Price *et al.*, 2002; Parpura *et al.*, 1994), connexin hemichannel related (Ye *et al.*, 2003), anion transporter related (Jeremic *et al.*, 2001) or via P_2X_7 receptor channels (Duan *et al.*, 2003). Analoguous to ATP release, these mechanisms appear to have different calcium sensitivities: vesicular release and anion transporter related release is calcium-dependent (Jeremic *et al.*, 2001; Parpura *et al.*, 2000) but release through hemichannels or P_2X_7 channels could not be blocked by

intracellular calcium buffering or emptying calcium stores with thapsigargin (Duan *et al.*, 2003; Ye *et al.*, 2003). Calcium-dependency has also been reported for glutamate release triggered by metabotropic glutamate receptor activation (Muyderman *et al.*, 2001) and for prostaglandin-related glutamate release (Bezzi *et al.*, 1998). Glutamate acts on ionotropic and metabotropic glutamate receptors activating calcium entry or calcium mobilization from stores respectively (Glaum *et al.*, 1990; Monaghan *et al.*, 1989; Nakahara *et al.*, 1997; Pearce *et al.*, 1986). Both ionotropic and metabotropic receptor activation triggers calcium responses in hippocampal astrocytes in brain slices (Porter *et al.*, 1995; Shelton *et al.*, 1999) and in Bergmann glia in cerebellar slices (Muller *et al.*, 1992; Shao *et al.*, 1997; reviewed by Gallo *et al.*, 2000), opening up the possibility that released glutamate produces on its turn calcium signals and is thus actively involved in the cell-to-cell communication of calcium signals in astrocytes and other glial cells.

Calcium signal communication between astrocytes thus seems to rely on many communication systems and messengers, acting either in parallel or displaying regional or cellular specialization (e.g. as demonstrated by Newman, 2001). The role of communicated calcium signals in astrocytes is currently not known, but probably depends on the signaling pathway being solicited and the brain region considered. Several functions have been proposed, either related to the calcium signal or to the messenger involved, including a role in astrocyte proliferation and differentiation (Fam *et al.*, 2003; Gallo *et al.*, 2000), in clearing neurotransmitters and ions from the extracellular space (De Pina-Benabou *et al.*, 2001; Newman, 1986; Porter *et al.*, 1995), in coordinating metabolic reactions (Tsacopoulos *et al.*, 1997), in brain cell volume regulation (Phillis *et al.*, 2002) and in spreading depression which is one of the models proposed to be involved in migraine pathophysiology (Basarsky *et al.*, 1998).

1.4.2.2 Calcium signals between neurons and astrocytes

Recent data have demonstrated that neurons and astrocytes exchange calcium signals both *in vitro* as well as in brain slices. The first evidence for communicated calcium signals was provided by Dani and coworkers (1992) who described astrocytic calcium changes in response to neuronal activity in cultured brain slices. Calcium signal communication has also been demonstrated in the inverse direction, i.e. from astrocytes to neurons in co-cultures (Charles, 1994; Nedergaard, 1994; Parpura *et al.*, 1994). Initially it was suggested that astrocyte-neuron calcium signal communication proceeded via gap junctions (Nedergaard, 1994) but a role for such junctions now seems to be reserved for the early stages of brain development (Froes *et al.*, 1999; Rozental *et al.*, 2001a). In the adult brain, the topic of astrocyte-neuron gap junctions is still controversial because their density is possibly below the detection limit and functional evidence is difficult to obtain because of the interposed low conductance pathway formed by neurites and astrocyte extensions (as in neuron-neuron coupling - Schmitz *et al.*, 2001). In certain systems however, e.g. the locus

ceruleus, neuron-glial gap junctions have been demonstrated in adult brain (Alvarez-Maubecin *et al.*, 2000). Further studies show that the bidirectional calcium signal communication system is mainly carried by extracellular messengers released from neurons or astrocytes. Various transmitters such as glutamate, acetylcholine, GABA, ATP or NO, released by neurons through calcium-dependent exocytosis, trigger calcium signals in cortical astrocytes (Pasti *et al.*, 1997), in hippocampal astrocytes (Bezzi *et al.*, 1998; Cornell-Bell *et al.*, 1990; Pasti *et al.*, 1997; Porter *et al.*, 1996) and in Bergmann glia (Matyash *et al.*, 2001). In addition, glutamate and ATP released from astrocytes, also by calcium-dependent mechanisms, trigger calcium changes in neurons in mixed cortical cultures (Araque *et al.*, 2000; Bal-Price *et al.*, 2002; Bezzi *et al.*, 1998; Hassinger *et al.*, 1995; Parpura *et al.*, 1994; Pasti *et al.*, 1997; Pasti *et al.*, 2001; Queiroz *et al.*, 1999), in hippocampal cultures (Araque *et al.*, 2000; Bal-Price *et al.*, 2002; Bezzi *et al.*, 1998; Hassinger *et al.*, 1995; Parpura *et al.*, 1994; Pasti *et al.*, 1997; Pasti *et al.*, 2001; Queiroz *et al.*, 1999) and in hippocampal or cortical slices (Araque *et al.*, 2000; Bal-Price *et al.*, 2002; Bezzi *et al.*, 1998; Hassinger *et al.*, 1995; Parpura *et al.*, 1994; Pasti *et al.*, 1997; Pasti *et al.*, 2001; Queiroz *et al.*, 1999). Because the signal flow is bidirectional, it has been proposed that astrocytes are part of a feedback circuit that starts with neuronal activity-induced astrocytic calcium signals, feeding back to the synapse through astrocytic glutamate release that modulates synaptic signal transmission (Araque *et al.*, 1999; Kang *et al.*, 1998; Newman *et al.*, 1998; Pasti *et al.*, 1997). Several recent reviews discuss glial-neuronal signaling in all detail (Araque *et al.*, 2001; Bezzi *et al.*, 2001a; Bezzi *et al.*, 2001b; Fields *et al.*, 2002; Haydon, 2001; Perea *et al.*, 2002; Vesce *et al.*, 2001; Zonta *et al.*, 2002). Clearly, glutamate exerts a wide range of effects in the brain, either by acting on receptors with subsequent changes in membrane potential or intracellular calcium concentration or by its uptake through specialized astrocytic transporter proteins (GLAST or GLT-1; Voutsinos-Porche *et al.*, 2003) thereby modulating glucose metabolism via changes in intracellular sodium concentration (Pellerin *et al.*, 1994). This last mechanism has been identified as the trigger for neurometabolic coupling which acts to adapt astrocytic glucose metabolism to the local neuronal demands (Magistretti *et al.*, 1999a).

1.4.2.3 Calcium signals between astrocytes and microvascular cells

Astrocytes are intermediately positioned between neurons and brain vessels, both in contact with their stellate extensions, and therefore occupy a key signaling position between these two important players (Attwell, 1994; Paspalas *et al.*, 1998). Since neurons can exchange calcium signals with astrocytes, the next question in row is whether astrocytes further propagate this signal to the vessels. Astrocytes are in contact with smooth muscle cells of arterioles, which determine the vessel diameter and thus blood flow, and with endothelial cells of capillary vessels, which form the blood-brain barrier where important transports take place. Work in astrocyte-endothelial co-cultures has demonstrated that calcium signals can be communicated between astrocytes and

endothelial cells in a bidirectional way, making use of both paracrine ATP signaling and gap junctions (Braet *et al.*, 2001; Leybaert *et al.*, 1998; Paemeleire *et al.*, 2000a). Recent work in acute brain slices has demonstrated endothelial calcium responses associated with electrically triggered calcium astrocytic waves (unpublished observation). Further work is needed to clear up the question concerning the presence of gap junctions –even at very low density- between astrocytes and endothelial cells. Paracrine ATP signaling between both cell types is another option as both astrocytes and endothelial cells can be stimulated to release ATP and are both endowed with purinergic receptors. Similar to astrocytes, endothelial cells release ATP either in a vesicular manner (Bodin *et al.*, 2001a) or via connexin-related mechanisms such as hemichannels (Braet *et al.*, 2003b; Braet *et al.*, 2003c; Leybaert *et al.*, 2003).

Brain endothelial cell lines communicate calcium signals between each other via gap junctional and paracrine signaling (Braet *et al.*, 2003b; Vandamme *et al.*, 2003), opening up the possibility of calcium signal conduction along brain blood vessels. Endothelial calcium signals can, in principle, be further communicated to the immune cells in the blood. Both gap junction channels and connexin hemichannels have been implicated in such communication system which has been dubbed 'the immunological synapse' (Oviedo-Orta *et al.*, 2002) and the target immune cells express the necessary purinergic receptors to respond to endothelially released ATP (Di Virgilio *et al.*, 2001a). Endothelial cells release large amounts (1-2 % of the total cell content) of ATP in response to a single $InsP_3$ stimulus (Braet *et al.*, 2003a), possibly to overcome the diluting effect of the blood flow. In this context, connexin hemichannels offer an attractive option as a pathway for fast and massive ATP release towards the blood. Finally, endothelial calcium signals might communicate back to the astrocytes (Braet *et al.*, 2001; Leybaert *et al.*, 1998) via the pathways discussed, and might also indirectly affect the neural tissue: work originally performed in the liver demonstrated that endothelially released NO can modulate calcium signal communication in surrounding cells (Charles, 1999), an observation that stresses the need to incorporate the microvascular cells in a picture of integrated neural tissue functioning.

Several possibilities should be considered concerning the role of astrocyte-endothelial calcium signal communication. Changes of endothelial calcium are considered a key step in disrupting the tight junctions between endothelial cells and opening of the blood-brain barrier (Abbott, 1998; Mayhan, 2001; Revest *et al.*, 1991; Tiruppathi *et al.*, 2002) and astrocyte-endothelial calcium signals might thus be involved in the process of barrier opening under pathological conditions. A fundamental question is whether endothelial calcium signals have effects on the transports occurring over the blood-brain barrier. We have put forward the hypothesis that astrocyte-endothelial calcium signals are instrumental in what we propose to call 'neurobarrier coupling' which, in concerted action with neurovascular and neurometabolic coupling, contributes to adapting the transport of glucose over the barrier to the local astrocytic and neuronal needs. There

have been reports on stimulation of the GLUT-1 glucose transporter in response to a calcium challenge with ionophores (Mitani *et al.*, 1995), but clearly, more physiologic calcium changes are needed to investigate this option. Preliminary work suggests that certain neurotransmitters acting on endothelial calcium are able to stimulate glucose uptake in endothelial cells (Braet *et al.*, 2000a) and recent work by Loaiza *et al.* (2003) has explored this possibility at the level of astrocytes where glutamate was shown to stimulate glucose uptake. The effect of glutamate on endothelial cells need to be determined but there is controversy whether endothelial glutamate receptors are really functional (Morley *et al.*, 1998). Recent work indicates that glutamate can induce carbon monoxide release from brain vessel endothelial cells (Leffler *et al.*, 2003).

Astrocytes are, with their typical endfeet, also in contact with arteriolar smooth muscle cells and calcium signals between these two cell types may be a crucial signaling step in neurovascular coupling, i.e. adapting the blood flow to the local neuronal demands. Recently, Zonta *et al.* (2003) have used brain slices to demonstrate that astrocytic calcium signals induced by electric stimulation of the neural tissue dilates small vessels in the vicinity through prostaglandin-dependent signaling. The role of calcium changes in target endothelial or smooth muscle cells was however not investigated (Anderson *et al.*, 2003). In acute brain slices, we observed responses in smooth muscle cells following electrically triggered astrocytic calcium waves (unpublished observation). Instead of a direct communication pathway between astrocytes and smooth muscle cells, endothelial cells might act as an intermediary unit in the signaling cascade. Released substances from endothelial cells can either elicit cerebral artery dilation or contraction (Kis *et al.*, 1999). Among the best known vasoconstrictory molecules are endothelins and the prostaglandin $PGF_{2\alpha}$, both being released in response to a calcium increase and able to induce calcium-mediated contraction of smooth muscle cells (Neylon, 1999; Saito *et al.*, 1989). The release of vasodilating agents such as NO and the prostaglandins PGE_2 and PGI_2 is also triggered by a calcium increase (Luckhoff *et al.*, 1988), but their relaxating effect on smooth muscle cells relies on a decrease of cytoplasmic calcium levels in response to cGMP (Goligorsky, 1988; Griffith *et al.*, 1985; Lee *et al.*, 1990). In addition to the former agents, vasoconstriction can also be induced by hyperpolarization of the smooth muscle cells, a response attributed to the calcium-dependent secretion of endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al.*, 1990). Calcium signal communication has furthermore been reported in the inverse direction, i.e. from smooth muscle to endothelial cells, a signal that is proposed to be mediated by gap junctions between both cell types and implicated in a feedback loop involving endothelial nitric oxide generation and smooth muscle cell relaxation (Dora *et al.*, 1997). An attractive hypothesis is that endothelial cells communicate calcium signals between each other from the capillary level to the arterioles, to cause dilation of upstream located resistance vessels (Faraci *et al.*, 1998; Iadecola, 1993). Capillary-to-arteriole endothelial signaling has been demonstrated outside the brain where the communicated signal

appears to be electrical for long range signaling and possibly calcium for short range signaling (Dora *et al.*, 2003; Sarelius *et al.*, 2000).

1.4.2.4 Calcium signals between astrocytes and microglia

The first evidence for calcium-mediated intercellular signaling between astrocytes and microglia in co-culture was provided by Verderio *et al.* (2001). In addition, stimulation of astrocytes in brain slices evoked calcium waves that spread to neighboring microglia (Schipke *et al.*, 2002). In both conditions, ATP was demonstrated to bring the calcium message from astrocytes to microglial cells by acting on P_2X_7 receptors. Several suggestions have been made concerning the function of astrocyte-microglial signals, again either related to the calcium signal or to the ATP messenger, including microglial activation (Inoue, 2002), control of the number of microglial cells under pathophysiological conditions (Verderio *et al.*, 2001) or a signal leading to microglial ramification and cell death (Wollmer *et al.*, 2001). An interesting option is that stimulation of microglial purinergic receptors leads to the release of the cytokine IL-1β (Sanz *et al.*, 2000) which reduces gap junctional communication and potentiates paracrine communication of calcium signals between astrocytes (John *et al.*, 1999a), a pathway through which microglia might modulate astrocyte communication. The ATP messenger does not have to come from astrocytes only, as neuronal ATP have also been reported to affect microglia and induce chemotaxis (Honda *et al.*, 2001; Kanazawa *et al.*, 2002). Another possible messenger is platelet-activating factor (PAF), which is released by neurons in a calcium-dependent manner and acts on microglial cells to increase calcium by mobilization from stores and entry form the extracellular space, a signal that again induces chemotaxis (Aihara *et al.*, 2000; Righi *et al.*, 1995).

1.4.2.5 Calcium signals between astrocytes and meningeal cells

Bidirectional calcium signaling has been described between astrocytes and meningeal cells (Grafstein *et al.*, 2000) where both the involvement of gap junctions and a purinergic mechanism have been implicated. Anatomically, a meningoglial network has been demonstrated (Mercier *et al.*, 2000) but *in vivo* work is necessary to confirm astrocyte-meningeal calcium signaling.

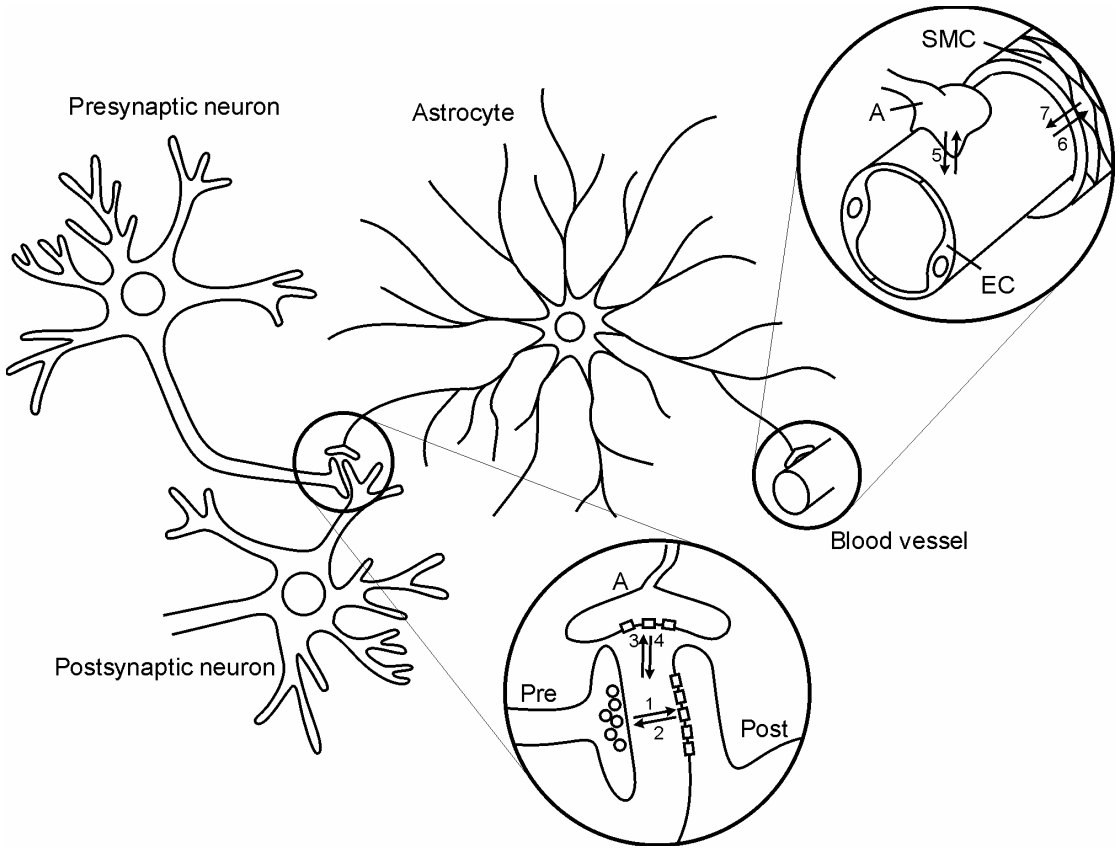

Fig. 1.5 *Schematic representation of calcium signal communication systems in the central nervous system. (1) Presynatically (Pre) released glutamate induces calcium changes in the postsynaptic (Post) nerve ending (Connor et al., 1994; Segal, 1995). (2) Postsynaptic NO triggers calcium increases in the presynaptic nerve ending and regulates neurotransmitter release (Reyes-Harde et al., 1999). (3) Release of glutamate and NO from neurons brings about calcium changes in astrocytes (A) ((Bezzi et al., 1998; Cornell-Bell et al., 1990; Pasti et al., 1997; Porter et al., 1996). (4) Astrocytes release ATP and glutamate causing calcium signals in neurons (Araque et al., 2000; Bal-Price et al., 2002; Bezzi et al., 1998; Hassinger et al., 1995; Parpura et al., 1994; Pasti et al., 1997; Pasti et al., 2001; Queiroz et al., 1999) and modulating synaptic activity (Araque et al., 1999; Kang et al., 1998; Newman et al., 1998; Pasti et al., 1997). (5) Bidirectional calcium signal communication has been demonstrated between astrocytes and endothelial cells (EC) in co-culture (Braet et al., 2001; Leybaert et al., 1998; Paemeleire et al., 2000a). (6) Vasoactive molecules released by endothelial cells through calcium-dependent mechanisms modulate smooth muscle cell (SMC) calcium concentration to produce contraction (Neylon, 1999; Saito et al., 1989) or relaxation (Luckhoff et al., 1988). (7) Smooth muscle cells provide a feedback calcium signal to endothelial cells (Dora et al., 1997). Finally, astrocytic calcium signals dilate small bloodvessels by paracrine messengers acting on smooth muscle cells, either directly or via interposed endothelial cells (Zonta et al., 2003).*

Chapter

2

Aim of the study

The research of the Calcium Signaling Group is directed towards the mechanisms of calcium signal communication between brain cells and the role of these signals in the communication between neurons and the brain vessels. Arteriolar brain vessels control the blood flow while capillary vessels are the site of the blood-brain barrier where important transports take place. While the cellular and molecular mechanisms that link neuronal activity to increased blood flow are only beginning to be understood (Zonta *et al.*, 2003), almost nothing is known concerning neuronal influences on, and signaling pathways to the endothelial cells of the blood-brain barrier. It is known from work in brain slices that calcium signals can be triggered in astrocytes by neuronal activity (Dani *et al.*, 1992). The starting point of this work was to determine how calcium signals can be communicated further along this pathway, i.e. from astrocytes to the endothelial cells.

What are the mechanisms of astrocyte-endothelial calcium signal communication?

Previous work, in various cell types, has suggested that intercellular calcium signals are communicated directly via gap junctions or indirectly via a biological messenger that is released by the cell and acts on receptors of neigboring cells (paracrine pathway) (Rottingen *et al.*, 2000). The aim of the work presented in **Chapter 4** was to determine the contribution of these two pathways in the communication of calcium signals between astrocytes and endothelial cells. This study was performed on astrocyte-endothelial co-cultures making use of microscope calcium imaging techniques, photoactivation of inactive calcium messengers and appropriate pharmacological tools.

What is the role of $InsP_3$ in paracrine purinergic signaling and is it involved in triggering cellular ATP release?

In **Chapter 5** we investigated the role of $InsP_3$ and calcium in paracrine purinergic calcium signal communication. The approach was twofold: we first examined the relative contribution of the intracellular/gap junctional pathway versus the paracrine purinergic pathway by studying the effect of pharmacological inhibitors on $InsP_3$-triggered calcium wave propagation. In addition, we investigated whether $InsP_3$, and its subsequent calcium increase, was able to trigger cellular ATP release.

What are the mechanisms of cellular ATP release?

Although ATP has gained much interest as an extracellular messenger, the mechanism of ATP

release is still a matter of debate. Several mechanisms have been suggested, including vesicular (exocytotic) release, transporter-related release or, as most recently reported, release by diffusion through connexin hemichannels (Abraham *et al.*, 1993; Bodin *et al.*, 2001a; Cotrina *et al.*, 1998a; Sauer *et al.*, 2000; Sprague *et al.*, 1998; Stout *et al.*, 2002). In **Chapter 6** we addressed the question whether ATP release occurs through connexin hemichannels, by investigating the effect of two different connexin mimetic peptides on triggered ATP release using two different triggering stimuli. A series of various pharmacological agents, reported to act on ATP release, was applied to the endothelial cell line GP8. In addition, we performed work on connexin transfected and connexin-free cell lines. Finally, we tested modulation of connexin hemichannels both at the level of ATP release and of uptake of reporter dyes into the cells.

What is the influence of TNF-α on the communication of calcium signals between blood-brain barrier cells?

Both TNF-α and calcium play a key role in opening the blood-brain barrier, a hallmark of the pathophysiological events in neuroinflammatory diseases (de Vries *et al.*, 1996; Petito *et al.*, 1992). We hypothesize that calcium signal communication between endothelial cells of the blood-brain barrier may act to spatially propagate the opening of the barrier. In **Chapter 7** we addressed the question whether TNF-α could influence the communication of calcium signals among endothelial cells. We investigated the influence of the cytokines on intercellular calcium waves and on cellular ATP release triggered by various stimuli, including the $InsP_3$ stimulus. In addition, we tested whether the cytokine influenced the triggered uptake of reporter dyes into the cells and affected gap junctional coupling.

What intracellular $InsP_3$ concentrations and extracellular ATP amounts are involved in our experimental work?

In **Chapter 8** we attempted to quantify the orders of magnitude of the increase in intracellular $InsP_3$ concentration attained with photoactivation of inactive $InsP_3$, and also to obtain an idea of the amounts of ATP released in the extracellular space in response to the $InsP_3$ stimulus. We determined the efficiencies of the various steps involved in the photoactivation protocol, investigated the dose-response relation between photoactivation light and ATP release and used both data sets to construct a dose-response relation between $InsP_3$ concentration and ATP release.

Chapter

3

Materials and methods

3.1. Cell cultures

3.1.1. Primary astrocytes

Primary rat brain astrocytes used in chapter 4 were isolated from neonatal rat cortices (postnatal day 1 or 2) following an adaptation of the method described by McCarthy *et al.* (1980). Cortical tissue fragments were dissociated using trypsin and repeated trituration through a pasteur pipette. The dissociated cells were filtered through a nylon mesh (120 and 20 µm mesh size) and seeded in 50 ml Falcon flasks in Basal Medium Eagle's (BME) supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine (all products from Gibco, Merelbeke, Belgium) and 20 mM glucose. Cells were grown to confluency over 7 to 10 days and the cultures were then enriched in type-1 astrocytes using a shaking procedure to remove overlaying process-bearing cells.

3.1.2. Primary endothelial cells

BCECs used in chapter 4 were isolated and cultured as described by Paemeleire *et al.* (1999b). Briefly, the brains of 2-3 month old Wistar rats were removed, the meninges peeled off and the cerebral cortex cut into small pieces. Tissue fragments were enzymatically (collagenase and DNAse from Sigma, Bornem, Belgium) and mechanically dissociated, and capillary fragments were isolated by density centrifugation and a second enzymatic dissociation (trypsin from Sigma). The resulting cell suspension was plated onto glass bottom microwell dishes coated with collagen type IV (Sigma) and fibronectin (Gibco). Capillaries were allowed to sediment and attach for 1-2h, and the remaining debris or cell-clumps were washed away by a subsequent medium change. Cultures were grown in DMEM-10 % FBS with 0.5 % heparin, 1 % Minimal Essential Medium non-essential amino acid solution (Sigma), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco), mixed at a ratio of 1:1 with astrocyte-conditioned BME-FBS.

3.1.3. Endothelial cell lines

In this study we used three rat brain endothelial cell lines:

RBE4 (used in chapter 5, 6 and 7) was a kind gift of Dr. F. Roux (Roux *et al.*, 1994) (Neurotech, Evry, France).

GP8/3.9 (used in chapter 6 and 7) was a kind gift of Dr. J. Greenwood (Greenwood *et al.*, 1996) (Dept. of Ophthalmology, University College of London, London, UK). Both cell lines were grown in alpha-MEM/Ham’s F10 (1:1) (Gibco) with 10 % fetal bovine serum (FBS), 1 µg/mL bFGF

(Boehringer Mannheim, Brussels, Belgium) and 30 mg/mL geneticin (Gibco).

SV-ARBEC (used in chapter 5) was a kind gift of Dr. D. Stanimirovic (Stanimirovic *et al.*, 1996) (Institute for Biological Sciences, NRC-CNRC, Ottawa, Canada) and was grown in Medium-199 with 10 % FBS, 100 mg/L peptone (Sigma), BME amino acids (Sigma) and BME vitamins (Gibco).

All endothelial cells were grown on glass bottom microwells (MatTek Corporation, Ashwood, MA, USA) coated with collagen type I (Boehringer Mannheim) and used for experiments upon confluency. For experiments in chapter 7, TNF-α was added to the culture medium and applied to the cells incubated under culture conditions (humidified 5 % CO_2) for the time periods used (2h or 24h). Matched control cultures were treated with culture medium not containing TNF-α in the same manner as the ones that received the cytokine.

3.1.4. Other cell lines

ECV304 cells (used in chapter 4, 5 and 8) were originally described as endothelial cells from human umbilical vein (Takahashi *et al.*, 1990) (European Collection of Animal Cell Cultures, Salisbury, UK), have now been shown to be epithelial bladder cancer cells (Dirks *et al.*, 1999). ECV304 cells are interesting in that they have a purinergic receptor pharmacology that is similar to primary brain capillary endothelial cells (Albert *et al.*, 1997; Brown *et al.*, 2000; Conant *et al.*, 1998; Howl *et al.*, 1998; Ohata *et al.*, 1997; Zhang *et al.*, 1997) and they develop tight junctions and a low permeability barrier in co-culture with glial cells making them useful in blood-brain barrier models (Hurst *et al.*, 1996; Ramsohoye *et al.*, 1998). They were grown in Medium-199 with 2 mM glutamine and 10 % FBS (Gibco).

Clone 9 cells (European Collection of Animal Cell Cultures) contain connexin-43 as the major connexin (Berthoud *et al.*, 1993) and were used as a positive control for the immunoblottings in chapter 5. They were grown in DMEM/Ham's F12 (1:1) with 2 mM glutamine and 10 % FBS (Gibco).

The HeLa parental and Cx43- and Cx32-transfected HeLa (used in chapter 6) were kindly provided by Dr. K. Willecke (Elfgang *et al.*, 1995) and were grown in MEM with 10 % FBS, and 1 µg/mL puromycin (Sigma) was included in the medium for the transfected HeLa cells.

3.2. Agents and buffers

Fluo-3 acetoxymethyl ester (fluo-3 AM), D-myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (NPE-caged $InsP_3$), o-nitrophenyl EGTA acetoxymethyl ester (NP-EGTA AM), adenosine 5'-triphosphate P3-1-(2-nitrophenyl)ethyl ester disodium salt (NPE-caged ATP), DMNB-caged fluorescein dextran (MW 3000), BAPTA acetoxymethyl ester (BAPTA AM), propidium iodide (PI, MW 668), tetramethylrhodamine dextran (MW 10000), fluorescein dextran (MW 70000), 6-carboxyfluorescein (6-CF, MW 376) and 5-carboxyfluorescein diacetate (5-CFDA) were obtained from Molecular Probes (Leiden, The Netherlands). Probenecid, apyrase grade III, 18-α-glycyrrhetinic acid (α-GA), pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) adenosine 5' trisphosphate disodium salt (ATP), $GdCl_3$, $LaCl_3$, niflumic acid, flufenamic acid, glibenclamide, NPPB, quinine, tumor necrosis factor alpha (TNF-α) were purchased from Sigma (Bornem, Belgium). Suramin hexasodium salt was from RBI (Natick, MA). The connexin mimetic peptides gap 26 (VCYDKSFPISHVR), gap 27 (SRPTEKTIFII) and des 5 (LEGHGDPLHLEEC) (Boitano *et al.*, 2000) were synthesized by solid phase chemistry and purified by HPLC and purity (95 %) assessed by HPLC. All peptides were kindly provided by Prof. W.H. Evans.

Normal HBSS-HEPES contained 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.18 mM Na_2HPO_4, 0.95 mM $CaCl_2$, 0.81 mM $MgSO_4$ and 25 mM HEPES. Ca^{2+}- and Mg^{2+}- free HBSS-HEPES contained 5.36 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.16 mM $NaHCO_3$, 0.313 mM Na_2HPO_4 and 25 mM HEPES. 5.55 mM glucose was added just before usage.

A stock solution of the products was prepared in deionized water except fluo-3 AM, NP-EGTA AM, BAPTA AM, 5-CFDA, α-GA, niflumic acid, flufenamic acid, glibenclamide and NPPB which were dissolved in dimethylsulfoxide (DMSO; Sigma). Final dilutions were prepared on the day of the experiment from aliquots. DMSO was without any significant effect at the concentrations used in the experiments. All experiments were performed at room temperature.

3.3. Calcium imaging

Cytoplasmic free calcium was monitored in chapter 4, 5 and 7 with epifluorescence microscopy and digital imaging using the calcium-sensitive probe fluo-3 (Fig. 3.1). Cell cultures were loaded with 10 µM fluo-3 for 1 hour at room temperature in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4) 1 mM probenecid, followed by 30 minutes de-esterification. Cells were viewed with an inverted epifluorescence microscope (Nikon Eclipse

TE300, Analis, Ghent, Belgium) using a x40 oil immersion lens (CFI Plan Fluor, Nikon). Fluo-3 fluorescence images were obtained by excitation at 485 nm, reflection of a dichroic mirror with cut-off at 505 nm, and emission bandpass filtering at 535 nm (485DF22, 505DRLPXR and 535DF35 filters respectively from Omega Optical, Brattleboro, VT). Images were captured using a silicon-intensified target camera (Cohu, San Diego, CA), an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) or a cooled integrating CCD (SensiCam, PCO, Kelheim, Germany) and were stored on an optical memory disk recorder (Panasonic TQ-2026F), an S-VHS video recorder (Panasonic, Avicom, De Pinte, Belgium) or directly to a PC equipped with an image acquisition and processing board (Data Translation, type DT3155, Marlboro, MA). The figures illustrating calcium imaging experiments are given as either changes of fluo-3 fluorescence, i.e. $\Delta F = F_t - F_0$ (with F_0 the fluorescence before the stimulus and F_t the fluorescence at time points after the stimulus) or as relative fluorescence changes, i.e. $\Delta F/F_0$.

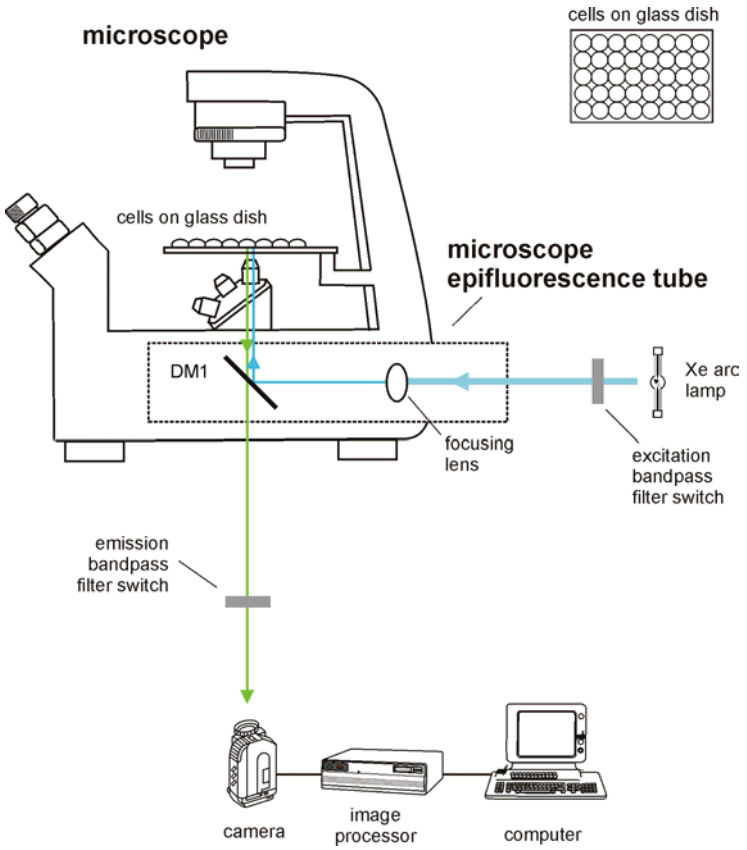

Fig. 3.1 *The microscope system used for calcium imaging. The excitation light is provided by either a Xe arc lamp, bandpass filtered at 485 nm and enters the microscope epifluorescence tube to produce a uniform field of illumination. The epifluorescence dichroic mirror (DM1; transmission cutoff at 505) reflects the short-wavelength excitation light to the specimen. The long-wavelength fluorescence emitted by fluo-3 in the cells (wavelength ~535 nm) passes through DM1 and is captured by an intensified or integrating CCD camera. Captured images are digitized with an image processor and are processed on the computer to display relative fluorescence changes.*

3.4. Caged compound loading

3.4.1. Caged $InsP_3$

Caged $InsP_3$ loading in chapter 4, 5, 6, 7 and 8 was done by electroporation. Electroporation loading was performed on the microscope stage using a special electrode consisting of two parallel wires supported by a holder made from inert delrin® acetal resin (Fig. 3.2A). Cultures were briefly rinsed with a special electroporation buffer with low electrical conductivity (300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1 mM $MgCl_2$ and 2 mM HEPES, pH 7.20) and thereafter a small volume (5 µl) of caged $InsP_3$ (200 µM) in electroporation buffer was added. The effective concentration of caged $InsP_3$ was approximately 100 µM because the added solution was diluted by a factor of approximately two because of the remaining fluid above the cell surface.

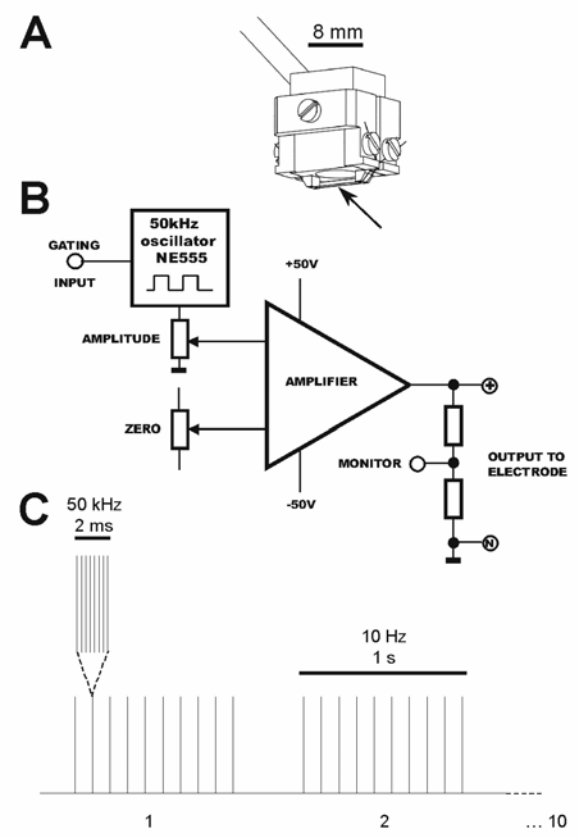

Fig. 3.2 *Electroporation setup to load the cells with caged $InsP_3$. A. The electroporation electrode consists of two parallel Pt-Ir wires (arrow) placed 400 µm apart (wire diameter 120 µm). The opening in the electrode holder (circular opening above wires) allows the passage of illumination light so that it can be positioned under microscopic control approximately 250 µm above the cells. B. Schematic drawing of the electrode driver circuit. The signal from a 50 kHz oscillator circuit (NE555 astable oscillator) is amplified to a signal amplitude that can be adjusted between 0 and 50 V. The gating input receives a TTL signal from a stimulator unit to produce repeated trains of 50 kHz pulses. C. The pulse train protocol used in the experiments. The voltage amplitude was 20 V giving an electric field strength of 500 V/cm (wire separation 400 µm).*

Electroporation was done with a 50 kHz positive going signal applied in a pulsed manner as illustrated in Fig. 3.2C. After electroporation, cultures were washed with HBSS-HEPES and left 20 min before the start of the experiment. The electroporation solution also contained dextran-rhodamine (100 µM) to visualize the electroporation zone and to estimate the efficiency of the loading procedure. This was done by comparing the dextran-rhodamine fluorescence in the

electroporation zone with the fluorescence of a thin layer of the same solution sandwiched between two coverslips. The thickness of the layer was approximately the thickness of the monolayer cell cultures, which was determined by inspection of fluo-3 loaded cultures with a confocal laser scanning microscope and found to be in the order of 20-25 µm.

The zone with caged $InsP_3$-loaded cells spreads out beyond the electroporation zone because caged $InsP_3$ (MW 635) is able to diffuse via gap junctions towards neighboring non-electroporated cells. The effective diffusion zone was determined to be 150 µm as illustrated in Fig. 8.6. In the work presented in chapter 8, it was necessary to know the dilution caused by this diffusion effect. We therefore applied an additional dilution factor of 800 (width of the electroporation zone in µm) divided by 800+150 (total width of caged $InsP_3$-loaded zone in µm).

3.4.2. Caged calcium

Caged calcium loading in chapter 4 and 5 was done by ester loading the cells with 5 µM NP-EGTA-AM for 10 minutes in 1 mL HBSS-HEPES containing probenecid (1 mM), followed by 30 minutes de-esterification (all at room temperature).

3.5. UV illumination

3.5.1. UV spot illumination

UV spot illumination was performed to trigger intercellular calcium waves in response to photolytic liberation of caged $InsP_3$, using two setups. In the first setup (Fig. 3.3), spot illumination was performed with an Hg-arc lamp coupled to the microscope epifluorescence input through a dichroic mirror with cut-off at 400 nm (400DCLP02, Omega Optical, Brattleboro, VT) and focused to the field diaphragm plane by means of two plano-convex fused silica lenses (SPX046 and SPX049, Newport, Leuven, Belgium). The UV beam was bandpass filtered at 330 nm (330WB80, Omega Optical, Brattleboro, VT) and the exposure time (0.2 s) was controlled by a mechanical iris diaphragm shutter (Wollensak, Rochester, NY).

The UV spot had a half-energy diameter of 10 µm, as determined by flashing a thin layer of DMNB-caged fluorescein dextran at 3 mM mixed (1:1) with Dako Glycergel (Dako Corporation, Carpinteria, CA). Regular inspection of the UV spot was done by illuminating a thin layer of Lucifer Yellow solution (as illustrated in Fig. 5.1H). To determine whether the UV spot only covered a single cell, we overloaded the cultures with NP-EGTA (10 µM NP-EGTA-AM for 60 minutes),

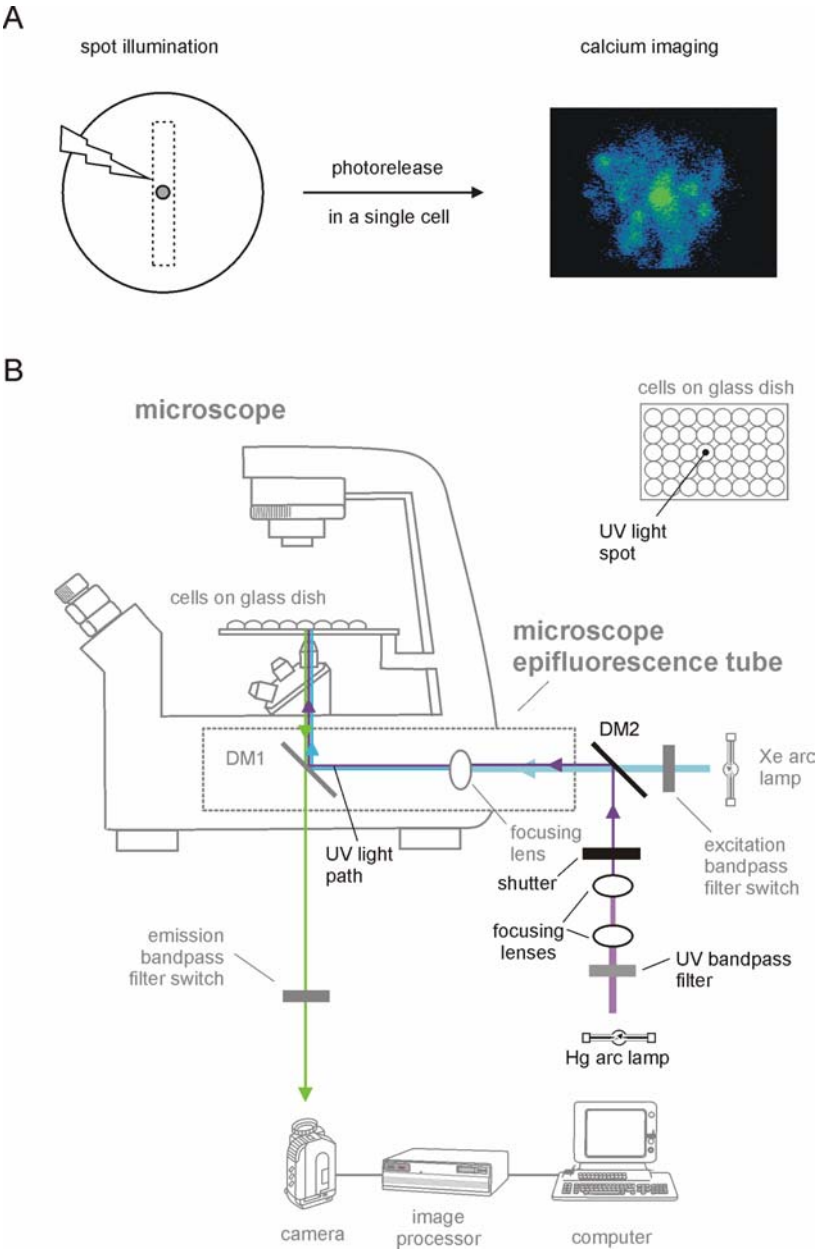

Fig. 3.3 *The microscope system adapted for UV spot illumination. A. Experimental setup. Cell cultures loaded with caged InsP3 by electroporation (rectangular zone delineated by the dashed line) were exposed to spot UV illumination (grey dot in the middle of the cell culture) directed to a single cell to trigger calcium changes that propagate from the stimulated cell to neighboring cells as an intercellular calcium wave (image at the right). B. Optical setup. UV excitation light is provided by a Hg arc lamp (horizontal bulb) and is bandpass filtered (330 nm), focused to a small spot with two plano-convex lenses and passed through a shutter. The UV light enters the epifluorescence tube by reflection off DM2.*

resulting in heavy calcium buffering in all the cells loaded because of the high calcium affinity of NP-EGTA in its caged non-photolyzed form. UV exposure liberates calcium only in the illuminated cell, while non-illuminated cells cannot display any propagated calcium signal because of effective

buffering by non-photolyzed NP-EGTA. Applying this kind of protocol showed that the photolytic UV light was only directed to a single target cell.

The second setup is illustrated in Fig. 3.4. BS1 in this figure was a 70 % transmission - 30 % reflection mirror and BS2 a 30 % transmission - 70 % reflection mirror (XF122 and XF123 resp., Omega Optical, Brattleboro, VT).

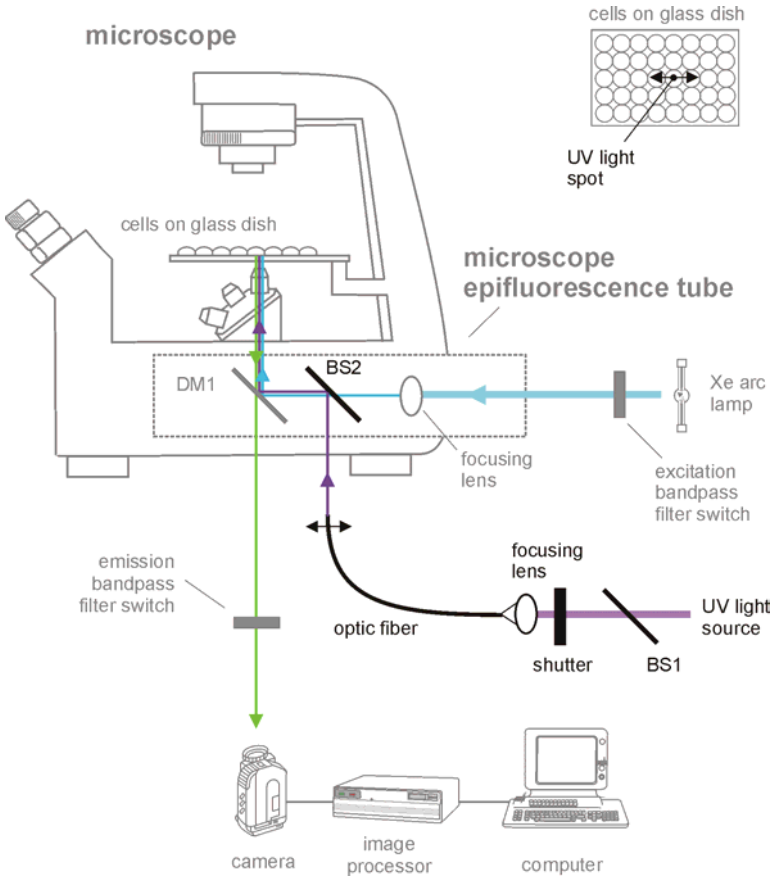

Fig. 3.4 *Second UV spot illumination setup for delivering photolytic UV light to a single cell. UV light from a laser source is coupled to a single mode optic fiber by means of a focusing lens. The UV light traverses a beam splitter (BS1). The microscope epifluorescence tube is adapted such that a second beamsplitter (BS2) is inserted close to the dichroic mirror in the microscope (DM1). The aperture of the optic fiber is positioned to form a focused image at the level of the cells on the glass dish. In this way, the fiber aperture appears as a spot (UV light spot) with a size inversely proportional to the magnification factor of the objective lens. The position of the spot in the cell field can be changed (bidirectional arrow on glass dish) by moving the fiber aperture laterally (bidirectional arrow at optic fiber). The duration of the UV exposure is determined by the shutter. The epifluorescence excitation light and the fluorescent light pathways illustrate the standard epifluorescence configuration.*

The optic fiber was a single mode fiber with a diameter of 200 µm; in combination with a x40 objective lens (Nikon S fluor 40x NA 1.3 oil) this gives a spot size of 5 µm diameter. The UV light source was either a flash lamp system (type JML-C2, Rapp OptoElectronic, Hamburg, Germany)

or the 351 nm UV line from an argon laser (type 2018, Spectra-Physics, Eindhoven, The Netherlands). All experiments were performed on Nikon Eclipse TE300 microscopes. Other valuable alternatives for spot illumination are discussed in Parpura *et al.* (1999a).

3.5.2. UV field illumination

UV field illumination was used to photorelease $InsP_3$ in a large zone of caged $InsP_3$-loaded cells, in order to obtain a measurable amount of ATP released by the cells in response to this stimulus. Light from a 100 W Hg-arc lamp with quartz collector lens (LH-M100C-1, Nikon) was bandpass-filtered at 330 nm (330WB80, Omega Optical, Brattleboro, VT), reflected by a mirror (10D510AL.2, Newport, Leuven, Belgium) and focused to the culture thereby exposing a large zone of cells (Fig. 3.5). The efficiency of photoliberating caged compounds by field illumination was investigated by exposing a thin layer of caged ATP (1 µM) to the photolytic UV light and measuring the amount of photoliberated ATP with a bioluminescent ATP assay kit (see ATP measurements). In chapter 8, we needed to know the attenuation of the photolytic UV light caused by passage of the light through the cell monolayer. This was determined by using power measurements on the transmitted light with a UV power meter (type 1830-C with 818-UV detector, Newport).

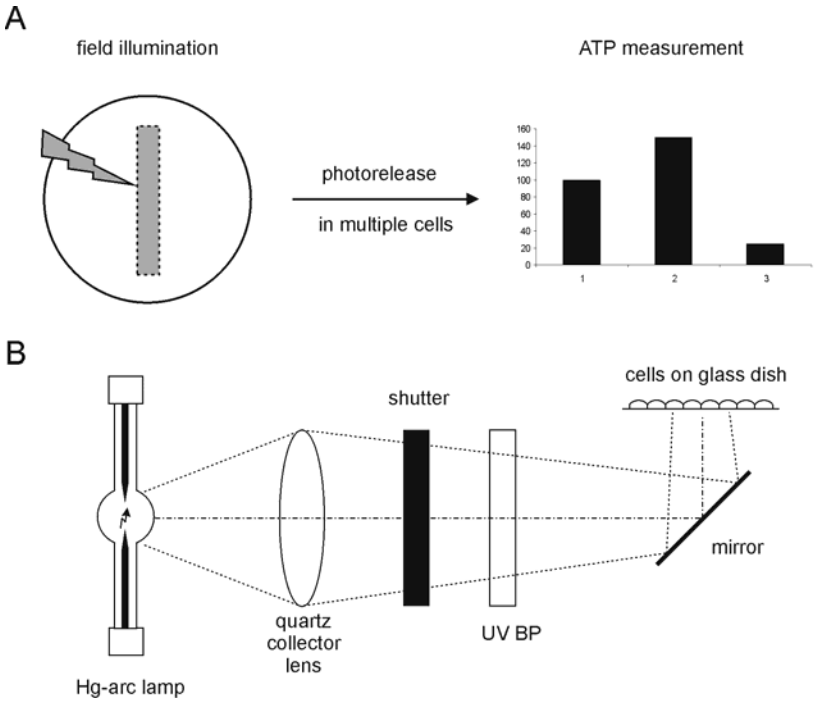

Fig. 3.5 *UV field illumination for delivering photolytic UV light to multiple cells. A. Experimental setup. Exposure of caged $InsP_3$–loaded cell cultures (dashed rectangle) to field UV illumination (gray zone) was used to measure ATP released by the cells in response to this stimulus. B. Optical setup. A focused image of the light arc of a Hg-arc lamp is formed at the level of the cells on a glass dish by means of a quartz collector lens. The light passes an electronically controlled shutter and a UV bandpass filter (UV BP).*

3.6. Extracellular ATP measurements

Cellular ATP release was determined in chapter 5, 6, 7 and 8 by using a bioluminescent luciferin/luciferase assay kit (FL-AA, Sigma, Bornem, Belgium). ATP release was triggered by two different protocols: either by photoliberating $InsP_3$ or calcium inside the cells (chapter 5, 6, 7 and 8) or by 2 minutes exposure of the cells to a zero extracellular calcium solution (Ca^{2+}- and Mg^{2+}-free HBSS-HEPES containing 1 mM EGTA; Sigma) (chapter 6 and 7). In both cases 100 µl of 200 µl supernatant was collected at the end of the trigger protocol and transferred to 100 µl ATP assay mix solution (used at 5-fold dilution; containing 1 mM EDTA). Light emission from the 200 µl mix was then measured with a custom build luminometer consisting of a photomultiplier tube (9924B, Thorn-Emi Electron Tubes, Middlesex, UK) mounted in a light-tight housing with a sample holder. Luminescence was quantified as photon counts within a time window of 100 milliseconds and average photon count values were calculated from 100 repeated measurements on the same specimen (10 seconds measurement time per sample). Mechanical movement of the cell cultures during manipulation was strictly minimized as small shocks to the cultures or solution changes produced by themselves measurable ATP release (as reported by Abraham *et al.,* 1997). Calibration curves with ATP were performed in a concentration range of 50-1000 nM.

To make the experimental conditions of the $InsP_3$-triggered and the control cell culture groups comparable, all cultures used in these experiments received UV field illumination, which was applied just before collection of the supernatant. Baseline (non-triggered, spontaneous) ATP release was slightly but not significantly affected by exposure to the photolytic UV light (100 % without illumination versus 88 $\pm$ 15 % with UV illumination; n=7; p=0.23). Some cell cultures in chapter 5 received mechanical cell stimulation, which served as a positive control condition because scratching the cultures liberates ATP from destroyed cells. The scratch was applied immediately after UV illumination and just before collection of supernatant. NP-EGTA loading and caged $InsP_3$ loading was done as described above (ester loading and electroporation loading respectively).

To ensure that the results obtained with the bioluminescent luciferin/luciferase assay were not altered by the pharmacological agents applied in this study, ATP calibration curves were also acquired in the presence of the concerned agents. Of the agents used, suramin was the only substance that had a significant inhibitory effect on the ATP calibration curves (Fig. 3.6). Therefore, suramin was not used in ATP assays experiments. All pharmacological agents were applied for a period of 30 minutes unless otherwise stated and were also present in the trigger solutions. Cell loading with the calcium buffer BAPTA was done before electroporation loading with caged $InsP_3$, by ester loading with 25 µM BAPTA-AM for 45 minutes in HBSS-HEPES containing probenecid (1 mM), followed by 30 minutes de-esterification (all at room temperature).

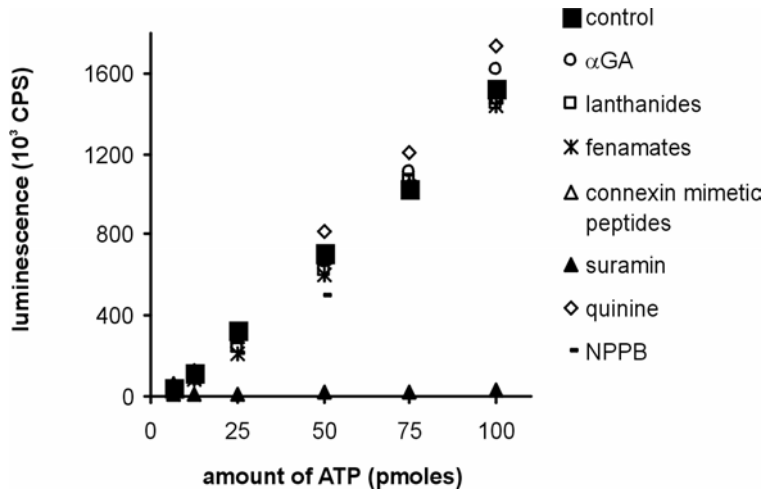

Fig. 3.6 *Effect of various inhibitor substances on the calibration curves of the luciferin-luciferase assay used to measure cellular ATP release. Luminescence is expressed as counts per second (CPS). The gap junction blocker αGA (50 µM), the fenamates (niflumic acid and flufenamic acid, 100 µM), connexin mimetic peptides (des 5 and gap 26, 0.25 mg/ml), the lanthanides (lanthanum and gadolinium, 50 and 30 µM) and quinine (100 µM) and NPPB (100 µM) did not interfere with the ATP assay, but the purinergic receptor inhibitor suramin (100 µM) completely abolished its response. All data points are the result of at least 3 experiments; the standard error is not shown for clarity. Only the calibration curve in the presence of suramin was significantly different form the control calibration curve.*

The composition of the zero calcium trigger solution was different when the lanthanides gadolinium and lanthanum were used in chapter 6, because EGTA has a high affinity for these ions thereby effectively complexing them. In that case it contained 100 µM EGTA, instead of the 1 mM normally used, and an increased lanthanide concentration (150 µM gadolinium and 200 µM lanthanum) to obtain free ion concentrations in the order of the concentrations used in non-EGTA containing solutions. Corresponding control experiments without lanthanides were also performed with this 100 µM EGTA trigger solution. Lanthanides had no effect on the ATP calibration curves because the assay kit used in our experiments contained EDTA to complex these ions (Boudreault *et al.*, 2002). Baseline release in zero calcium-triggered ATP release was determined by exposure to normal HBSS-HEPES solution and baseline release in $InsP_3$-triggered ATP release was performed with vehicle electroporated cells.

In the experiments using caged ATP (chapter 8), a thin layer of solution (5 µl sandwiched between two coverslips) was exposed to UV light and immediately thereafter mixed with the same quantity of ATP assay mix solution. The mixture was placed in the sample holder of the luminometer and luminescence was measured. The ATP results presented in chapter 8 as the fractional release of the total cellular ATP content were calculated taking into account a total cell surface on the glass bottom culture dish of 141 mm² (diameter 13.4 mm), a surface of caged $InsP_3$-loaded cells of 7.6 mm² (8 mm x 0.95 mm), a cell density of 1140 ± 200 cells/mm² (n=4) and a cellular protein content of 8.3 ± 1.4 ng protein per cell (n=4).

chapter

4

Astrocyte-endothelial calcium signals conveyed by two signaling pathways

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Abstract

Astrocytes and endothelial cells are in close contact with each other at the blood-brain barrier, where important molecular transports take place. Despite these key morphological and functional properties, little is known regarding the dynamic signaling processes that occur between these two cell types. We investigated astrocyte-endothelial calcium signaling mechanisms in a co-culture model prepared from primary rat cortical astrocytes and ECV304 cells. We used flash photolysis of caged inositol-trisphosphate ($InsP_3$) and gentle mechanical stimulation to trigger astrocyte-endothelial calcium signals and to investigate the underlying propagation mechanisms. Photolytically releasing $InsP_3$ in a single cell triggered increases in cytoplasmic calcium concentration that propagated between astrocytes and endothelial cells in either direction. These propagating calcium signals did not cross cell-free zones and were not affected by fast superfusion or by the purinergic inhibitors apyrase and suramin, indicating that they are communicated through an intracellular pathway in conjunction with gap junctions. Electrophysiological experiments confirmed a low degree of astrocyte-endothelial electrical cell-to-cell coupling. Mechanical stimulation of a single cell also triggered astrocyte-endothelial calcium signals, but in contrast to the former triggering mode, these signals overpassed cell-free zones and were significantly inhibited by apyrase, thus indicating the involvement of an extracellular and purinergic messenger. Astrocyte-endothelial calcium signaling also occurred in co-cultures prepared with astrocytes and primary rat brain capillary endothelial cells. We conclude that astrocytes and endothelial cells can exchange fast acting calcium signals (time scale of seconds) that can be communicated through an intracellular/gap junctional pathway and an extracellular purinergic pathway.

4.1. Introduction

Calcium signals can be communicated between cells by paracrine or gap junctional communication. In some cell types, e.g. astrocytes and endothelial cells, intercellular calcium signals take the form of a radially spreading wave of cytoplasmic calcium increase called an intercellular calcium wave (Charles, 1998; Sanderson, 1996). An interesting aspect of these calcium waves is that they display an apparent amplification effect: they are triggered by stimulation of just a single cell and this initiates propagation of the signal over a large population of cells. Thus, astrocytes are 'calcium excitable' brain cells allowing transmission of calcium signals over a chain of cells. Recent work has shown that astrocytic calcium signals can be communicated to neurons (Charles, 1994; Dani *et al.*, 1992; Murphy *et al.*, 1993b; Nedergaard, 1994), can influence synaptic functioning (Araque *et al.*, 1998a; Araque *et al.*, 1998b; Bacci *et al.*, 1999; Kang *et al.*, 1998) and can be transmitted to meningeal cells (Grafstein *et al.*, 2000). In study, we investigated the communication of calcium signals between astrocytes and endothelial cells.

Brain capillary endothelial cells form the actual boundary of the blood-brain barrier, because of the presence of intercellular tight junctions and a low degree of transcellular vesicular transport (Drewes, 1999). As a consequence, the molecular and ionic movements over the barrier are mediated by specific transporters, such as the GLUT-1 glucose transporter. Thus, blood-brain barrier endothelial cells form an interesting regulation point to modulate the transfer of molecules to and away from the brain. Despite the close relationship of astrocytes and endothelial cells and the key transport phenomena going on, little is known concerning the signaling pathways operating between these cells. Endothelial cells are endowed with many receptors and there is currently evidence for a role of interleukin-6, nitric oxide, angiotensin, ICAM and TNF-α in astrocyte-endothelial signaling (Brett *et al.*, 1995; Etienne-Manneville *et al.*, 2000; Kakinuma *et al.*, 1998; Murphy *et al.*, 1993a; Shafer *et al.*, 1997; Sporbert *et al.*, 1999; Takemoto *et al.*, 1994). Regarding calcium ions, the intra- and extracellular homeostasis and signaling mechanisms have been thoroughly investigated in the two cell types separately (Deitmer *et al.*, 1998; Himmel *et al.*, 1993; Nilius, 1998; Verkhratsky *et al.*, 1996a; Verkhratsky *et al.*, 1998). At the blood-brain barrier, calcium has since long been implicated as a messenger that increases the permeability (Abbott, 2000; Abbott *et al.*, 1991; Olesen, 1989), and many agonists acting through calcium (and PKC), like the bradykinin analogue RMP-7, increase the blood-brain barrier permeability (Bartus *et al.*, 1996; Doctrow *et al.*, 1994; Mackic *et al.*, 1999). Despite this evidence, little effort has been undertaken to investigate calcium signaling pathways in an experimental situation where astrocytes and endothelial cells are closely associated and able to interact. In the present work we investigated gap junctional and extracellular routes of calcium signaling in co-cultures prepared from astrocytes and endothelial cells.

4.2. Materials and methods

4.2.1. Cell cultures

Astrocyte-endothelial co-cultures were prepared from primary rat brain astrocytes and a spontaneously transformed endothelial cell line derived from human umbilical vein (ECV304, European Collection of Animal Cell Cultures, Salisbury, UK; Takahashi *et al.*, 1990), as described by Leybaert *et al.* (1998). Primary rat brain astrocytes were isolated from neonatal rat cortices (postnatal day 1 or 2) following an adaptation of the method described by McCarthy and de Vellis (McCarthy *et al.*, 1980). Cortical tissue fragments were dissociated using trypsin and repeated trituration through a pasteur pipette. The dissociated cells were filtered through a nylon mesh (120 and 20 µm mesh size) and seeded in 50 ml Falcon flasks in Basal Medium Eagle's (BME) supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine (all products from Gibco, Merelbeke, Belgium) and 20 mM glucose. Cells were grown to confluency over 7 to 10 days and the cultures were then enriched in type-1 astrocytes using a shaking procedure to remove overlaying process-bearing cells. ECV304 cells were grown in 50 ml culture flasks in Medium-199 with 2 mM glutamine and 10 % FCS (further called Medium-199-FCS; culture medium products from Gibco). Co-cultures were prepared by trypsinizing astrocyte and endothelial cell cultures and plating them simultaneously onto glass bottom microwell dishes (Mattek, Ashwood, MA, USA) at a ratio of 40 astrocytes to 1 endothelial cell in Medium-199-FCS. After 3 to 4 days, the co-cultures consitently had distinguishable zones of endothelial cells interspersed amongst the astrocytes. Co-cultures older than 4 days were not used because at that stage astrocytes started to invade the endothelial cell zones. Astrocyte identity was determined by immunostaining with Cy-3-conjugated antibodies to GFAP (Sigma, Bornem, Belgium). Endothelial cell identity was determined by their typical phase-contrast appearance, their GFAP-negativity, or by prelabeling the cells with the lipohilic carbocyanine dye CellTracker CM-DiI (Molecular Probes, Leiden, The Netherlands). Prelabeling was performed before co-culturing with astrocytes and consisted of 2 min incubation in 50 µM CM-DiI. GFAP-Cy-3 images were acquired with FITC excitation-emission settings while TRITC settings were used in conjunction with CM-DiI.

Some experiments were done on astrocyte-endothelial co-cultures prepared with primary rat brain capillary endothelial cells (BCECs) instead of the ECV304 cells. BCECs were isolated and cultured as described by Paemeleire *et al.* (1999b). Briefly, the brains of 2-3 month old Wistar rats were removed, the meninges peeled off and the cerebral cortex cut into small pieces. Tissue fragments were enzymatically (collagenase and DNAse) and mechanically dissociated, and capillary fragments were isolated by density centrifugation and a second enzymatic dissociation (trypsin). The resulting cell supsension was plated onto glass bottom microwell dishes coated with collagen

type IV (Sigma) and fibronectin (Gibco). Capillaries were allowed to sediment and attach for 1-2h, and the remaining debris or cell-clumps were washed away by a subsequent medium change. Cultures were grown in DMEM-10 % FCS with 0.5 % heparin, 1 % Minimal Essential Medium non-essential amino acid solution (Sigma), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco), mixed at a ratio of 1:1 with astrocyte-conditioned BME-FCS. Astrocyte-BCEC co-cultures were prepared by adding a low density of astrocytes to BCEC cultures, either at the stage where clearly distinguisable and non-confluent endothelial cell islands were still present or else after BCEC confluency and reorganization to a network of capillary-like structures (Laterra *et al.*, 1990).

4.2.2. Calcium imaging

Calcium waves were monitored with epifluorescence microscopy and digital imaging using the calcium-sensitive probe fluo-3. Co-cultures were loaded with fluo-3 by incubation for 1h at room temperature in Hanks' Balanced Salt Solution buffered with 25 mM HEPES (HBSS-HEPES) containing 10 µM fluo-3-AM (Molecular Probes, Eugene, OR), 0.05 % pluronic and 1 mM probenecid. This was followed by 1h of de-esterification in HBSS-HEPES at room temperature. Cells were viewed with an inverted Nikon Eclipse TE300 epifluorescence microscope using a ×40 oil-immersion lens. Fluo-3 fluorescence images were obtained by excitation at 480 nm, reflection off a dichroic mirror with a cut-off wavelength at 510 nm, and bandpass emission filtering at a center wavelength of 535 nm. Images were captured with a silicon-intensified target camera (Cohu, San Diego, CA) or an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) and were stored on an optical memory disk recorder (Panasonic TQ-2026F), an S-VHS videorecorder (Panasonic, Avicom, De Pinte, Belgium) or directly to a PC equiped with an image acquisition and processing board (Data Translation, type DT3155, Marlboro, MA). The figures illustrating calcium imaging experiments are given as either changes of fluo-3 fluorescence, i.e. $\Delta F = F_t - F_0$ (with F_0 the fluorescence before the stimulus and F_t the fluorescence at time points after the stimulus) or as relative fluorescence changes, i.e. $\Delta F/F_0$.

The size of an intercellular calcium wave at its maximal propagation state was determined by calculating the surface area of cells with $\Delta F/F_0 \geq 25$ %. The effect of pharmacological agents on intercellular calcium waves was assessed by measuring their effect on the surface area at maximal extension. The propagation speed of an intercellular calcium wave was obtained from the gradient of distance versus time-to-peak plots. Fast superfusate flow was applied by placing a superfusion source and suction pipette close to the borders of the imaged zone. Flow velocity was determined after the experiment from the movement of suspended fluorescent beads (Fluospheres, 1 µm diameter, Molecular Probes, Leiden, The Netherlands; 1 µl of a 2 % stock solution per 100 ml HBSS-HEPES). Apyrase (grade-III, Sigma) was diluted in HBSS-HEPES to a stock concentration

of 2000 IU/ml.

4.2.3. Caged IP_3 loading and flash photolysis setup

The cells were loaded with D-myo-inositol 1,4,5-trisphosphate, P4(5)-(1-(2-nitrophenyl)ethyl)ester trisodium salt (Molecular Probes, Eugene, OR or Calbiochem, La Jolla, CA), further called caged IP_3, by electroporation. Electroporation was performed on the stage of the microscope and using an electrode consisting of two parallel platinum/iridium wires separated 0.8 mm apart and positioned 200 μm above the cells. The electrical pulse protocol consisted of 4 repeats of ten 50 kHz bursts of 1 ms duration and at a field strength of 500 V/cm. During electroporation, the cells were kept in 10 μl of a poorly conducting medium composed of 300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1mM $MgCl_2$, buffered with 2 mM HEPES (pH=7.20) and containing 200 μM caged IP_3. After electroporation, the cultures were washed and kept in HBSS-HEPES. In some experiments Texas red dextran (MW 3000; Molecular Probes; 1.7 μM) was included in the electroporation medium in order to locate and visualize the zone of electroporated cells. The electroporation zone appeared as a strip-like band located underneath the electrode wires with a width in the order of 300-500 μm. Flash photolysis experiments were started 30 min after electroporation, and were done at a distance of approximately 100 μm away from the border of the electroporated zone. The UV light for flash photolysis was delivered by a Hg-arc lamp coupled to the microscope epifluorescence input by reflection off a dichroic mirror with cut-off at 400 nm and focused with a biconvex fused silica lens (focal distance 250 mm) to the field diaphragm plane. The UV beam was bandpass filtered at 330 nm (80 nm half-energy bandwidth) and the exposure time, ranging from 0.1 to 2 s, was controlled by a mechanical shutter (Uniblitz, Vincent Associates, Rochester, NY). The UV spot had a half-energy diameter of 7.1 μm, as determined by flashing a dried thin layer of caged fluorescein dextran (MW 3000; Molecular Probes). Details of the electroporation loading and flash photolysis protocols can be found in Leybaert *et al.* (2000).

4.2.4. Dye coupling

Gap junctional coupling of the cells was investigated by micro-injection or scrape loading the cultures with the gap junction permeable dyes Lucifer Yellow, 6-carboxyfluorescein or biocytin. For micro-injection, glass micropipettes with a tip diameter of < 1 μm were backfilled with a 5 % (w/v) solution of the dye in distilled water for Lucifer Yellow (dilithium salt, Sigma) and biocytin (Molecular Probes), or in 20 mM potassium citrate (pH=7.0) for 6-carboxyfluorescein (Molecular Probes). Cells were impaled and the micropipette was left for 10 min in place to allow the dye to diffuse into the cytoplasm. Epifluorescence images were acquired with FITC excitation-emission

settings for 6-carboxyfluorescein; for Lucifer Yellow the excitation wavelength was lowered to 420 nm. For biocytin, micro-injected cultures were fixed with 4 % formaldehyde in phosphate buffered saline (PBS) for 10 min and permeabilized for 30 min in 0.5 % (v/v) Triton X-100 in PBS. Cultures were then incubated with FITC labeled avidine 1/100 in PBS for 2h and images were acquired. For scrape loading, confluent astrocyte cultures were incubated for 10 min in a nominally calcium-free saline consisting of 137 mM NaCl, 5.36 mM KCl, 0.81 mM $MgCl_2$, 5.55 mM D-glucose and 25 mM HEPES (pH 7.4) containing 0.4 mM 6-carboxy fluorescein. Thereafter, a linear scratch was applied to the cells with a thin metal needle (Terumo, type 26G×½", Leuven, Belgium) and the culture was left for another 10 min in the same solution. Finally, cultures were washed three times with HBSS-HEPES and fluorescence images were acquired another 10 min later.

4.2.5. Electrophysiology

Electrical cell-to-cell coupling was measured by injecting DC-current into one cell and recording the resulting membrane potential changes in neighboring cells. Stimulating and recording electrodes were pulled from glass capillaries (filamented borosilicate glass, 0.68 mm inner and 1.2 mm outer diameter) to a resistance of 100-150 MΩ when filled with 3M K-acetate. Electrodes were connected to a current injection and a voltage recording amplifier (WPI, Hamden, CT; model M701 and M4-A respectively) and the bath ground was connected through a Ag/AgCl pellet. Current and voltage traces were recorded on a VCR data recorder (model 420, A.R. Vetter Co., Rebersberg, PA) and were analyzed using Pclamp software (Axon Instruments, Foster City, CA). After each experiment, the electrodes were withdrawn and the voltage changes induced by current injection were recorded with the electrodes in the bath to determine artifactual voltage changes (e.g. due to capacitative coupling).

4.2.6. Connexin immunostaining

Cell cultures were fixed for 20 min in 4 % formaldehyde in PBS and then incubated in PBS with 3 % BSA for 20 min, followed by 1 % glycine in PBS for 10 min; each of these steps was separated by three rinses with PBS. To block non-specific binding, cultures were incubated for 45 min in 10 % normal rabbit serum in PBS. Immunostaining was done overnight by incubation in mouse anti-connexin-43 (monoclonal antibody to connexin-43, ProBIO Ltd., Kent, England; dilution 1/3000) at 4°C, followed by five PBS rinses. Thereafter cultures were incubated with rabbit anti-mouse immunoglobulin-TRITC (1/60) for 45 min at room temperature and, after rinsing with PBS, mounted on glass slides with Dako mounting medium. Negative controls were done by the same procedure but with the primary antibody replaced by normal mouse serum.

4.2.7. Transmission electron microscopy

Cultures were fixed in 2 % glutaraldehyde in 0.1 M sodiumcacodylate (pH 7.4) containing 1 % $CaCl_2$ (w/v) at 4°C during 2h. The material was then rinsed in cacodylatebuffer with 7.5 % sucrose followed by osmication overnight at 4°C in 1 % OsO_4 in 0.1 M cacodylate with $K_3Fe(CN)_6$. Blockstaining with uranyl acetate (0.5 % in veronal acetate, pH=5) was carried out for 30 min at room temperature and the tissue was then embedded in LX (Ladd, Burlington, Vermont, USA). Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with a Jeol JEM-100B electron microscope (Jeol, Tokyo, Japan). In some cultures lanthanum nitrate (1 %) was added to the fixative, rinsing and osmication solutions.

4.2.8. Mechanical cell stimulation

Single cells were mechanically stimulated with a glass micropipette (patch pipette with a tip size of ~5 µm) attached to a piezoelectric device and micromanipulated to approximately 4 µm above the target cell. Activation of the piezoelectric crystal deflected the electrode tip over approximately 5 µm during 150 ms, thereby touching the cell in a gentle and non-disruptive way.

4.2.9. Statistical analysis

Data are expressed as mean ± SEM, with (n) denoting the number of experiments on different cultures. Statistical significance was tested using a t-test for unpaired observations and a p value less than 0.05 was considered as statistically significant.

4.3. Results

4.3.1. Astrocyte-endothelial co-cultures

The experiments were performed on astrocyte-endothelial co-cultures prepared from primary rat cortical astrocytes and endothelial ECV304 cells. All experiments were done at zones displaying clear confluent patches of endothelial cells with little endothelial cell contamination in the surrounding astrocyte region. The choice of a suitable zone was based on phase-contrast inspection and morphological recognition of the two cell types: ECV304 cells were distinguished from astrocytes by their larger size, clearly visible cell outlines, multiple nucleoli and typical cobble stone appearance. In some experiments endothelial cells were identified by prelabeling them with CM-DiI. Cell identity was further examined after the calcium imaging experiment by GFAP-immunostaining: astrocytes stain GFAP-positive while endothelial cell zones appear as GFAP-negative patches (Fig. 4.1).

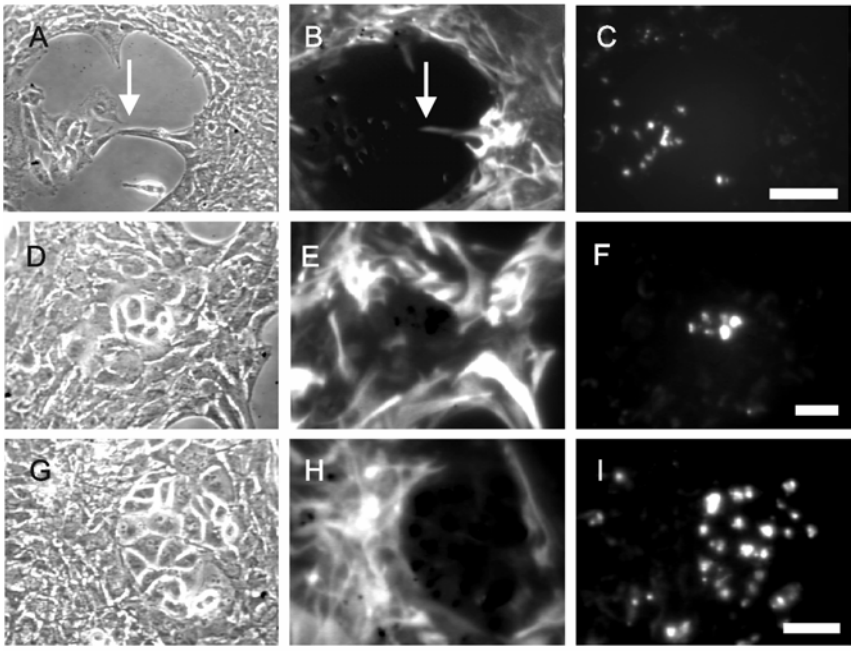

Fig. 4.1 *Astrocyte-endothelial co-cultures. A, B and C respectively illustrate phase-contrast, GFAP immunofluorescence and CM-DiI staining in a subconfluent astrocyte-endothelial co-culture. GFAP immunostaining identifies astrocytes while CM-DiI positive cells represent endothelial cells prelabeled with the dye. An astrocyte extension seeking contact with the endothelial cells can be distinguished in A and B (arrow). D, E and F and G, H and I illustrate similar image sequences recorded from confluent co-cultures. The CM-DiI fluorescent label and the GFAP-Cy3 immunoprobe are characterized by similar excitation-emission properties and the CM-DiI artifact was therefore substracted from the GFAP images. The bars measure 50 µm.*

4.3.2. Intercellular calcium waves triggered by flash photolysis of caged $InsP_3$

Exposure of a single astrocyte, in a culture previously loaded with caged $InsP_3$ by electroporation, to a focused beam of UV light increased cytoplasmic calcium in the exposed cell and triggered a calcium wave that propagated radially to the surrounding astrocytes (Fig. 4.2A). The distance traveled by the intercellular calcium wave depended on the duration of the UV exposure (Fig. 4.2B). The relation between the surface area of the calcium wave, i.e. the area of cells displaying a $\Delta F/F_0 \geq 25$ % at the moment of maximal wave extension, and the duration of UV exposure was a saturating function with a half-maximal value at 0.49 ± 0.09 s (n=5) exposure time. All further experiments were done with UV exposures (further called UV flash) of 1 s duration; with these settings the wave surface area at maximal extension was 6420 ± 952 μm^2 (n=5; this corresponds to a maximal wave radius of ~45 µm assuming circular wave morphology), and the wave propagation speed averaged 16.0 ± 1.98 µm/s (n=5).

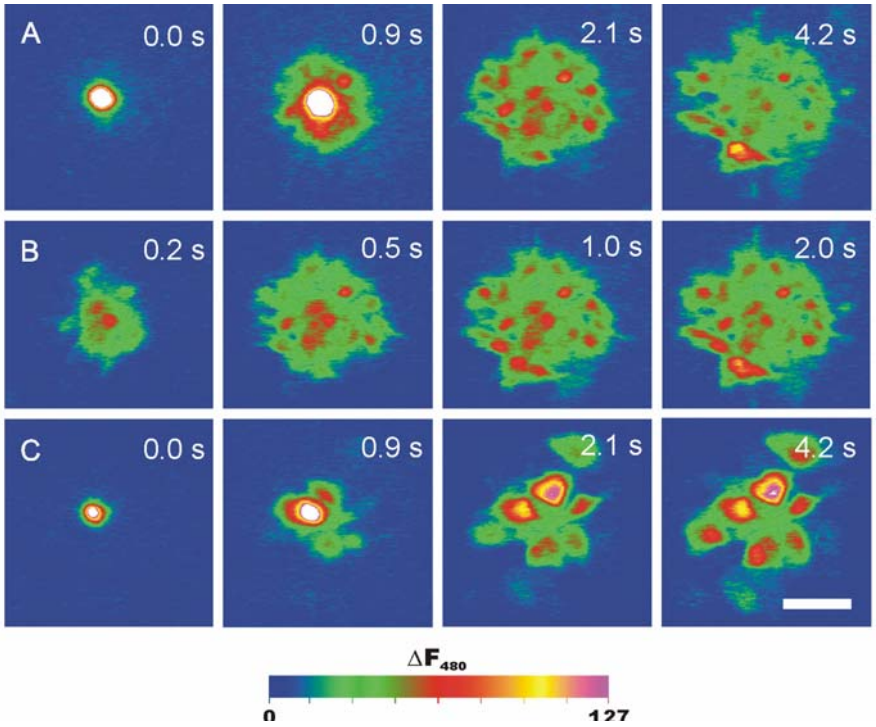

Fig. 4.2 *Flash photolysis of caged $InsP_3$ triggers intercellular calcium waves in astrocytes and endothelial cells. Changes in cytoplasmic calcium are represented by changes in fluo-3 fluorescence (ΔF480). A and C illustrate time sequences of intercellular calcium wave propagation in confluent cultures of astrocytes (A) and endothelial cells (C). In both sequences the time from the beginning of the UV flash is indicated in the upper right corner. The white spot visible in the first two images of A and C represents the fluorescence artifact produced by photolytic stimulation; the artifact appears larger than the actual size of the UV spot. B shows intercellular calcium waves (at maximal extension) in function of the duration of the triggering UV flash. The numbers in the upper right corners indicate the duration of the UV flash in this sequence. The bar measures 50 µm.*

When the UV flash was applied to astrocytes located away from the electroporated zone the surface area of the produced intercellular calcium waves decreased exponentially with the distance (space constant ~150 µm). No calcium responses were observed when the UV flash was applied to cell cultures not previously loaded with caged $InsP_3$. Application of the UV flash to a single endothelial cell within a zone of mainly endothelial cells produced endothelial intercellular calcium waves with a surface area of 3580 ± 780 μm^2 (n=5), which is significantly smaller than in astrocytes (p=0.025) (Fig. 4.2C). Wave propagation in endothelial cells was also significantly slower (p=0.006) than in astrocytes, with an average speed of 8.51 ± 1.16 µm/s (n=5).

4.3.3. $InsP_3$-triggered intercellular calcium waves and extracellular purinergic messengers

To test the possibility that $InsP_3$-triggered intercellular calcium waves propagate by an extracellular purinergic messenger, as has been demonstrated with mechanical or electrical cell stimulation (Cotrina *et al.*, 1998a; Cotrina *et al.*, 1998b; Guthrie *et al.*, 1999; Hassinger *et al.*, 1996), we examined the effect of fast superfusion flow, cell-free gaps and antagonists of purinergic signaling. The shape or size of $InsP_3$-triggered intercellular calcium waves in astrocytes was not influenced by fast superfusion (superfusate flow velocity of ~400 µm/s; n=5). $InsP_3$-triggered intercellular calcium waves were not able to bridge cell-free gaps of approximately 20 µm wide (n=5; Fig. 4.3). In the presence of apyrase (40 IU/ml, 8 min incubation), an ATP- and ADP- degrading enzyme (Guthrie *et al.*, 1999) $InsP_3$-triggered intercellular calcium waves were slightly but not significantly reduced (to 87.5 ± 17.8 % of the control size, n=37). By contrast, intercellular calcium waves triggered by mechanical stimulation of a single astrocyte were significantly ($p<0.0001$) reduced to 42.4 ± 9.1 % (n=10) by this protocol.

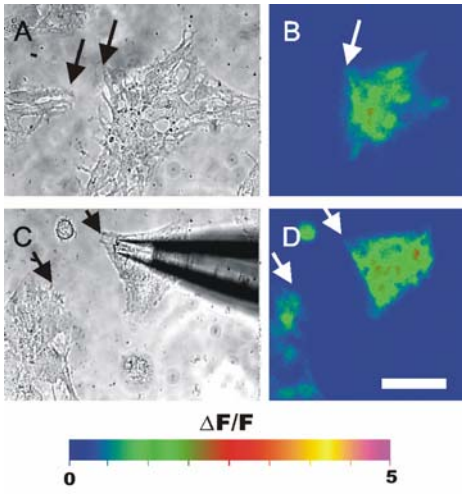

Fig. 4.3 *$InsP_3$-triggered intercellular calcium waves do not propagate across cell-free gaps. A shows a phase-contrast image of a subconfluent astrocyte culture with two confluent cell zones separated by a cell-free gap (arrows). B shows an intercellular calcium wave triggered by flash photolysis of caged $InsP_3$ (shown at maximal extension; calcium expressed as ΔF480/F480). $InsP_3$-triggered intercellular calcium waves were never observed to bridge a gap. C and D demonstrate a similar experiment but the calcium wave was triggered here by mechanical stimulation (pipette visible on phase-contrast). Calcium waves triggered by mechanical stimulation were able to cross the cell-free gap (arrows). The bar measures 50 µm.*

Suramin, a blocker of P_{2Y} purinergic receptors (Charlton *et al.*, 1996), did not significantly affect the size of $InsP_3$-triggered intercellular calcium waves (94.7 ± 17 % of the control size, n=24). Taken together, the absence of any effects of rapid superfusate flow, the inability to overpass small cell-free zones, and the non-significant effects of apyrase and suramin indicate that an extracellulary diffusing purinergic messenger is not involved in the cell-to-cell propagation of $InsP_3$-triggered calcium waves.

4.3.4. $InsP_3$-triggered astrocyte-endothelial calcium signals

When the UV flash was directed to an astrocyte located in the neighborhood of an endothelial cell island (1-2 cells away from the interface), the resulting astrocytic intercellular calcium wave crossed the astrocyte-endothelial interface and activated calcium changes in an endothelial cell (Fig. 4.4A); this was observed in 3/6 experiments and in all cases the endothelial response was limited to a single cell. Directing the UV flash to an endothelial cell located at the interface with the surrounding astrocytes triggered an endothelial intercellular calcium wave that propagated to the surrounding astrocytes (Fig. 4.4B). Endothelial to astrocyte calcium wave propagation was observed in 5/6 experiments and these waves involved 1-8 astrocytes.

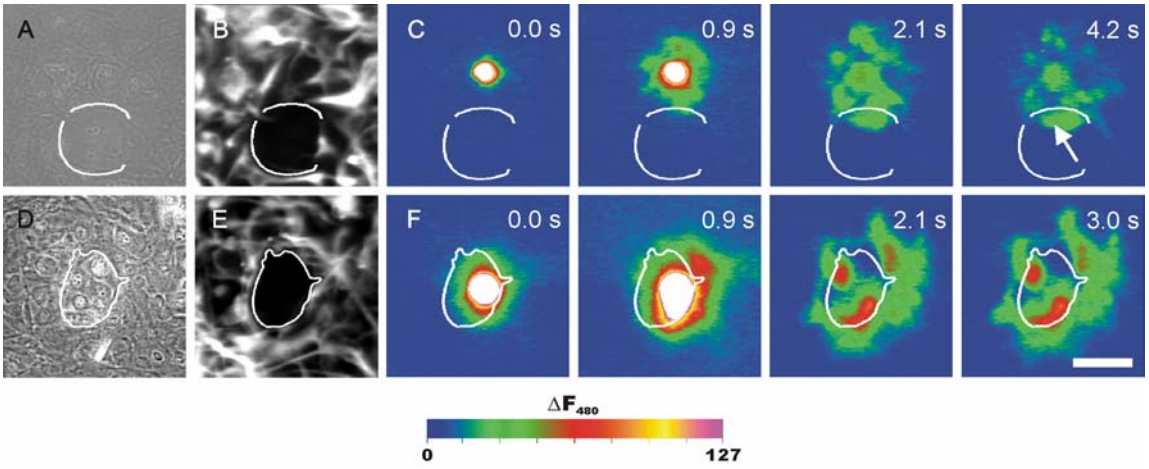

Fig. 4.4 *$InsP_3$-triggered intercellular calcium waves are communicated between astrocytes and endothelial cells in co-culture. A is a phase-contrast image and B a GFAP immunostaining of the corresponding zone. C is a sequence of calcium images (ΔF480). The photolytic release of $InsP_3$ in an astrocyte triggered an intercellular calcium wave that crossed the astrocyte-endothelial interface (white line) and evoked a calcium response in a single endothelial cell (arrow). D, E and F illustrate a similar experiment but the flash was applied here to an endothelial cell. This triggered calcium changes in the endothelial cells that propagated to multiple surrounding astrocytes. Note that the fluorescence artifact produced by photolytic stimulation of the endothelial cell is much larger than the actual size of the UV beam (10 µm at half-width) and is thus by no means indicative of the flash size. The bar measures 50 µm.*

4.3.5. Astrocyte-endothelial gap junctional coupling

The finding of $InsP_3$-triggered astrocyte-endothelial calcium signals together with the fact that these waves are not mediated by extracellular messengers suggests that the two cell types are connected by gap junctions. We investigated gap junctional coupling with fluorescent dyes Lucifer Yellow (LY), 6-carboxy fluorescein (CF) and biocytin. Micro-injection of LY in an astrocyte readily demonstrated dye coupling to neighboring astrocytes (space constant of exponential fluorescence decay of 182 $\pm$ 31 µm, n=5). By contrast, a much lower degree of dye coupling was found when endothelial cells were injected: dye spread to only a single adjacent endothelial cell was observed in 2/18 LY injections, 7/32 CF injections and 0/5 biocytin injections. No dye coupling was observed between astrocytes and endothelial cells by injecting either an astrocyte or an endothelial cell located close to the astrocyte-endothelial interface (n=5 for LY and 6 for CF injections in an astrocyte; n=10 for CF and 5 for biocytin injections in an endothelial cell) (Fig. 4.5).

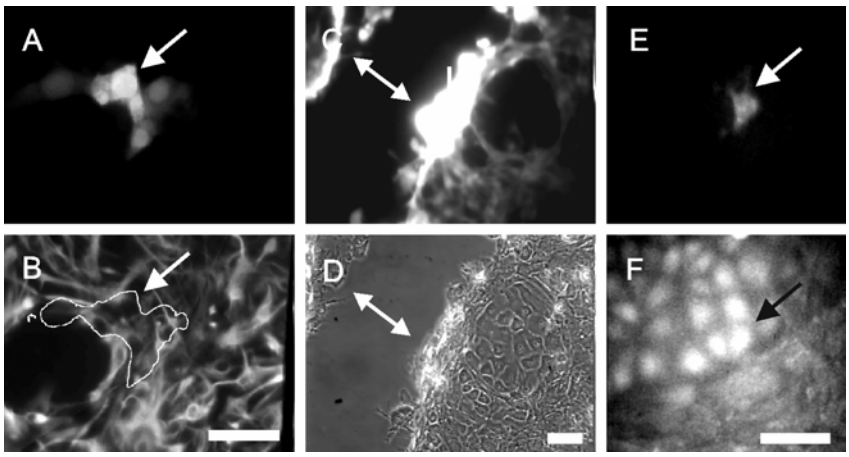

Fig. 4.5 *Astrocytes do not show dye coupling to endothelial cells in co-culture. A shows Lucifer Yellow (LY) fluorescence in astrocytes following micro-injection of an astrocyte (arrow) located close to the interface with the endothelial cells. B is the corresponding GFAP immunostaining. Dye spread occurred from the micro-injected astrocyte to surrounding astrocytes but not to endothelial cells which are located in the GFAP-negative (black) patch on the GFAP image. The white line on the GFAP image marks the position of LY positive astrocytes. C shows carboxy-fluorescein (CF) fluorescence in astrocytes following scrape loading and D is the corresponding phase-contrast image displaying the cell-free zone (arrow) following scraping and an endothelial cell island. Dye spread occurred among the astrocytes but the endothelial cell island did not take up any dye. E shows FITC-avidine labeled biocytin in a micro-injected endothelial cell (arrow) and F is a fluo-3 fluorescence image illustrating the position of endothelial cells (large and bright cells in the upper left half of the picture) and astrocytes (diffusely fluorescing cells in the lower right half). No dye spread occurred between endothelial cells or between endothelial cells and astrocytes. The bars measure 50 µm.*

Absence of dye coupling does not exclude gap junctional coupling (Zahs, 1998) and we further probed electrical cell-to-cell coupling using electrophysiological techniques. Astrocytes and endothelial cells had significantly ($p<0.0001$) different resting membrane potentials of -57.4 ± 3.1 mV (n=24) and -35.7 ± 2.4 mV (n=48) respectively. Injection of 10 nA current pulses (50 ms pulse duration, 10 Hz repetition frequency) in an astrocyte induced synchronous membrane potential changes averaging 3.02 ± 0.31 mV (n=11) in neighboring endothelial cells (Fig. 4.6). Similarly, injection of 10 nA current pulses in an endothelial cell caused membrane potential changes of 2.39 ± 0.22 mV (n=7) in adjacent astrocytes and of 3.79 ± 0.79 mV (n=7) in adjacent endothelial cells. Thus astrocytes are electrically coupled to closely located endothelial cells.

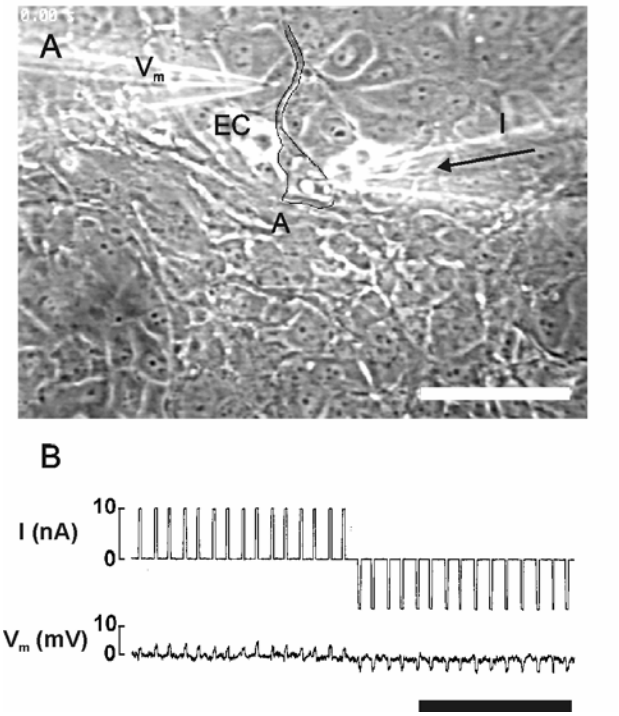

Fig. 4.6 *Astrocytes are electrically coupled to endothelial cells in co-culture. A shows the position of the microelectrodes on a phase-contrast image: an astrocyte (marked 'A') located close to the endothelial cells was impaled with a current electrode (I) while an endothelial cell (EC) approximately two cell diameters away was impaled with a voltage recording electrode (Vm). In these experiments, astrocytes were distinguished from endothelial cells by their morphological appearance and by their significantly more negative membrane potential. The astrocyte in this example showed a clear extension penetrating the endothelial cell zone and projecting towards the endothelial cell from which voltage recording was done; the contours of this cell and its extensions are marked by the thin black line. The traces in B illustrate that current injection in the astrocyte produces synchronous voltage changes in the endothelial cell. The bar in A measures 50 µm; the bar in B indicates time and represents 1 s.*

The presence of gap junctions was further examined by immunostainings with antibodies to connexin-43, which is the most abundant connexin in both astrocytes and endothelial cells (Giaume, 1996; Little *et al.*, 1995; Scemes *et al.*, 1998; Spray *et al.*, 1999; Zahs, 1998). Connexin-43 immunoreactivity was very intense in astrocytes, was also observed in endothelial cells, and was present at the interface between astrocytes and endothelial cells (Fig. 4.7).

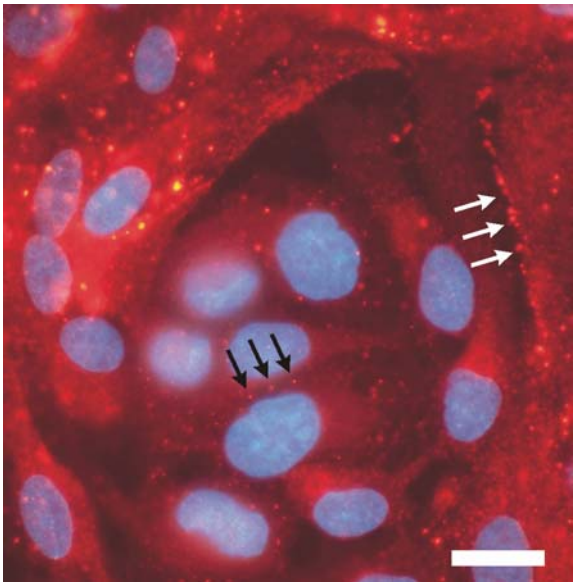

Fig. 4.7 *Immunostaining of an astrocyte-endothelial co-culture for connexin-43. Immunopositive dots are shown in red (TRITC), while cell nuclei are indicated in blue (DAPI staining). Connexin immunoreactivity was extensive in the astrocytes, most clearly in this figure at the astrocyte borders delineating the endothelial cell island (at the level of and below the white arrows). The endothelial cells (large 9 nuclei in the center of the image) were less marked, but clear immunopositive dots could be observed at the cell circumference (dark arrows). The bar measures 10 µm.*

4.3.6. Transmission electron microscopy of astrocyte-endothelial co-cultures

Electron microscopy was performed to examine the astrocyte-endothelial interface for the presence of gap junctions. ECV304 cells were easily recognized by their abundant cell organelles, a large number of nucleoli and a much denser cytoplasm; astrocytes were identified by their intermediate filaments that were most obvious in the processes (Fig. 4.8). At the astrocyte-endothelial interface, long sequences of adjoining plasma membranes of the two cell types were analyzed but we did never observe astrocyte-endothelial gap junctions. Astrocytes always showed extensive gap junctions between each other (Fig. 4.8B) but we could not document gap junctions between endothelial cells. Inspection of the interface furthermore demonstrated that the typical astrocyte end-feet extensions and a basal lamina, which are typical of *in vivo* preparations, were not present. Interestingly, we found numerous tight junctions and desmosomes between astrocytes in close proximity to endothelial cells (Fig. 4.8C); such membrane specializations have not been described so far between mammalian astrocytes (Landis *et al.*, 1983) but they are known to constitute the blood-brain barrier in lower animals (Abbott *et al.*, 1992). Tight junctions were also present between endothelial cells, and they were visibly more abundant than in monocultures of endothelial cells, as has been demonstrated in other co-culture models (Janzer *et al.*, 1987; Nagy *et al.*, 1991).

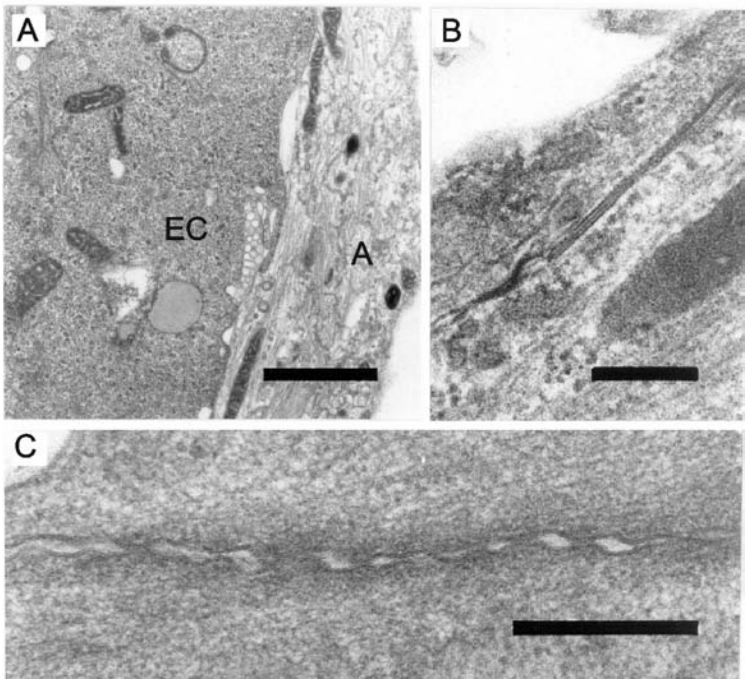

Fig. 4.8 *Electron microscopy of astrocyte-endothelial co-cultures. A illustrates the astrocyte-endothelial interface: the astrocyte (marked 'A') can be recognized by its abundant intermediate filaments (GFAP) while the endothelial cell (EC) is characterized by a dense cytoplasm. B demonstrates the typical pentalaminar configuration of a gap junction between two astrocytes. C illustrates a tight junction between astrocytes. The bar in A measures 2 µm; in B-C the bars are 0.2 µm.*

4.3.7. Intercellular calcium waves triggered by mechanical stimulation

To investigate the involvement of an extracellular purinergic pathway in astrocyte-endothelial calcium signaling, we initiated intercellular calcium waves in astrocytes and endothelial cells by gentle mechanical stimulation. Mechanical stimulation of a single astrocyte triggered intercellular calcium waves in the astrocytes with a surface area of 8580 ± 630 µm^2 (n=13), which is significantly larger than $InsP_3$-triggered intercellular calcium waves in astrocytes (p=0.043). The peak relative fluorescence change ($\Delta F/F_0$) in the responding astrocytes was significantly larger (p=0.0003) as compared to $InsP_3$-triggered intercellular calcium waves: 88.8 ± 2.5 % versus 76.3 ± 2.3 % respectively (n=13). In the endothelial cells, gentle mechanical stimulation triggered intercellular calcium waves with a surface area of 17130 ± 930 µm^2 (n=13; significantly larger than $InsP_3$-triggered intercellular calcium waves in endothelial cells with p=0.0001). In the presence of apyrase (40 IU/ml, 30 min incubation) the size of intercellular calcium waves was significantly reduced to 31.5 ± 8.6 % (n=13) of the control size for astrocytes and to 8.1 ± 1.1 % for endothelial cells (p<0.0001 for both) (Fig. 4.9).

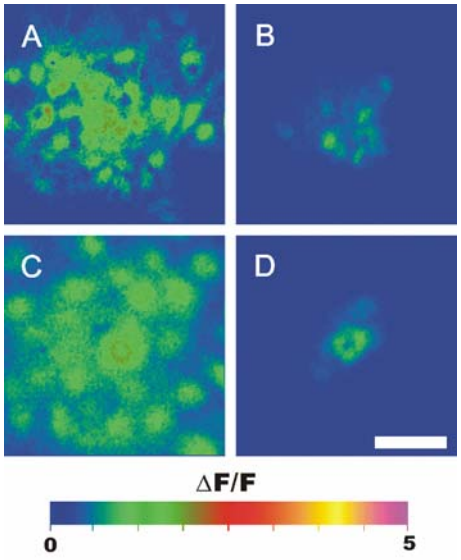

Fig. 4.9 *Astrocytic and endothelial intercellular calcium waves triggered by mechanical stimulation are largely reduced by apyrase. A shows an astrocytic intercellular calcium wave under control conditions and B illustrates a similar wave in the presence of apyrase (experiments on different cultures). Astrocytic calcium waves were reduced to approximately 1/3 of their control size in the presence of apyrase (40 IU/ml). C and D illustrate an endothelial intercellular calcium wave under control conditions (C) and in the presence of apyrase (D). Endothelial calcium waves were more strongly affected by apyrase and were reduced to approximately 1/12 of their control size. The bar measures 50 µm.*

4.3.8. Astrocyte-endothelial calcium signals triggered by mechanical stimulation

Mechanical stimulation of an astrocyte located 20-30 µm away from the astrocyte-endothelial interface triggered a calcium response in the astrocytes that propagated towards the endothelial cells, most often in a sequential way (Fig. 4.10A). In some experiments the endothelial calcium response did not occur in a sequential way; instead endothelial cells distant from the astrocytes responded first and endothelial cells proximate to the astrocytes responded later (Fig. 4.10B).

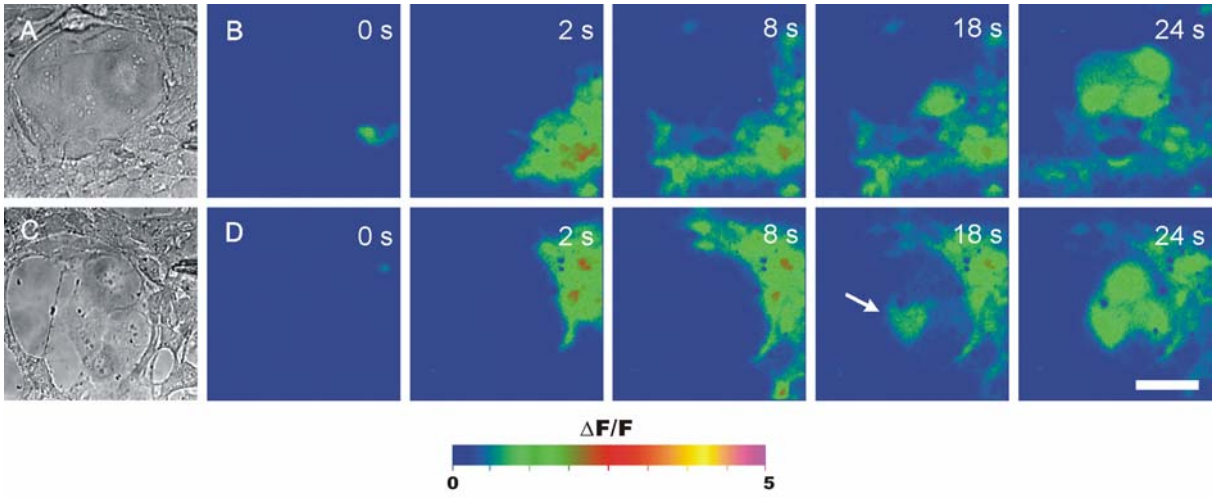

Fig. 4.10 *Astrocyte-endothelial calcium signals triggered by mechanical stimulation. A is a phase-contrast image and B shows the corresponding fluo-3 calcium image sequence. Mechanical stimulation of an astrocyte triggered an intercellular calcium wave that was further communicated to the endothelial cells. The times are indicated in the upper right corners. C and D illustrate a similar experiment as shown in A and B but the endothelial calcium response followed a slightly different pattern: the first responding endothelial cell (arrow) was located at some distance away from the astrocyte-endothelial interface, and the endothelial cells most closely located to the astrocytes responded thereafter. The bar measures 50 µm.*

The endothelial response to mechanical stimulation of an astrocyte, located on average 27.3 ± 2.0 µm (n=15) away from the astrocyte-endothelial interface, was characterized by an endothelial surface area of above threshold calcium change encompassing 3027 ± 546 μm^2 (n=15; ~5 endothelial cells responding on average) (Fig. 4.11A-B). In the presence of apyrase, stimulation of an astrocyte located at about the same distance from the interface (26.2 ± 3.3 µm, not statistically different from the distance used in the control experiments) triggered astrocyte-endothelial calcium waves with a significantly smaller ($p<0.0001$) size of endothelial response that was 30.2 ± 12.5 % of the control response (n=15; ~3 endothelial cells responding on average) (Fig. 4.11C-D). The peak $\Delta F/F_0$ in the responding endothelial cells was significantly smaller ($p<0.0001$) in the presence of apyrase: 47.7 ± 4.7 % versus 76.7 ± 3.8 % in control (n=15). These experiments demonstrate that astrocyte-endothelial calcium signals triggered by mechanical stimulation of an astrocyte are in part mediated by an extracellular apyrase-degradable messenger.

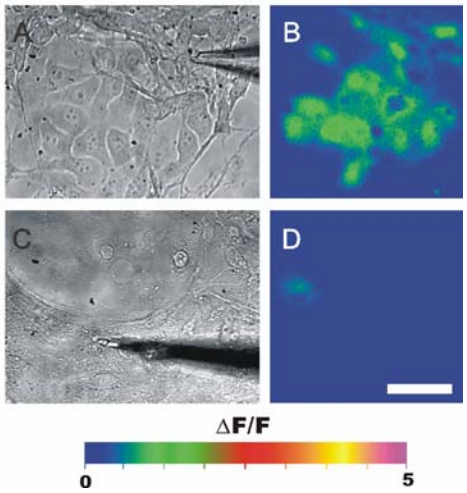

Fig. 4.11 *Astrocyte-endothelial calcium signals triggered by mechanical stimulation are largely reduced by apyrase. A is a phase-contrast image and B shows the astrocytic and endothelial calcium responses elicited by mechanical stimulation of an astrocyte (cell under the stimulation pipette) under control conditions. C and D illustrate a similar set of images acquired in a different co-culture and with apyrase (40 IU/ml) in the bath. On average, apyrase reduced the surface area of the endothelial calcium response to approximately 1/3 of its size under control conditions. The bar measures 50 µm.*

4.3.9. Astrocyte-endothelial calcium signals in co-cultures of astrocytes and primary brain capillary endothelial cells

We performed experiments on astrocyte-endothelial co-cultures prepared with endothelial cells from primary cultures of rat brain capillary endothelial cells (BCECs). Mechanical stimulation of an astrocyte located 25-30 µm away from the astrocyte-endothelial interface produced a calcium signal in the first row of BCECs (n=4; Fig. 4.12A-B). In some experiments, astrocyte seeding was delayed to the stage where the BCEC cultures developed a network of endothelial tubular structures, called 'capillary-like' structures by Laterra *et al.* (1990). These capillary-like structures encircled zones devoid of endothelial cells and when astrocytes were seeded at that stage they settled down in these cell-free zones. In co-cultures prepared in this way, mechanically triggered astrocytic calcium signals propagated towards the capillary-like endothelial arrangements and

extended over several tens of micrometers along these structures (n=5; Fig. 4.12C-D).

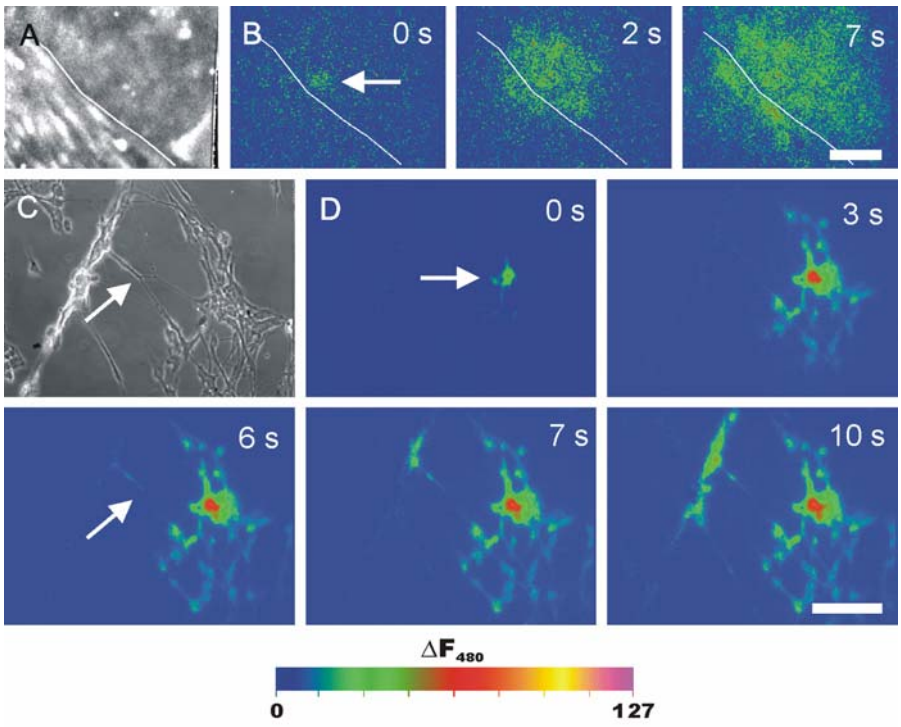

Fig. 4.12 *Astrocyte-endothelial calcium signals in co-cultures prepared with primary brain capillary endothelial cells (BCECs). A represents a raw fluorescence image after fluo-3 loading allowing to distinguish BCECs below the white line (typically aligned and spindle shaped cells) and astrocytes above the white line. B illustrates that gentle mechanical stimulation of an astrocyte (arrow) triggers an astrocytic calcium wave that extends into the first row of BCECs. C is a phase-contrast image of a second type of co-culture where BCECs were allowed to develop tubular capillary-like structures before astrocyte seeding (oblique phase bright chain of cells at the left). Note that some astrocytes send extensions towards the BCECs (oblique arrow in C). D illustrates that mechanical stimulation of an astrocyte (arrow) triggers an astrocytic calcium wave that follows the astrocyte extension (oblique arrow) and activates a calcium signal in the BCECs located at the end of the extension. The calcium signal then further propagates over the longitudinal BCEC structure. The bars measure 50 µm.*

4.4. Discussion

The present work was undertaken to investigate how astrocytes and endothelial cells exchange calcium signals. The main findings of our experiments, performed in astrocyte-endothelial co-cultures, are that astrocytes and endothelial cells can communicate calcium signals by either an intracellular $InsP_3$- and gap junction-dependent pathway, or by an extracellular ATP-dependent pathway.

Most experiments were done on astrocyte-endothelial co-cultures prepared with the ECV304 cell line, a spontaneously transformed cell line derived from human umbilical vein endothelium (Takahashi *et al.*, 1990). Recently, the endothelial origin of this cell line has been challenged (Brown *et al.*, 2000). The reason for choosing this cell line is that co-cultures prepared with these cells readily form, in contrast to co-cultures prepared with BCECs, a clearly distinguishable interface with the astrocytes (Paemeleire *et al.*, 1997; Paemeleire *et al.*, 1999b). In addition, ECV304 cells in co-culture with glial cells on diffusion membranes develop a low permeability state typical of the blood-brain barrier (Hurst *et al.*, 1996; Ramsohoye *et al.*, 1998). Furthermore, their expression and pharmacology of purinergic receptors is identical as in primary BCECs (Albert *et al.*, 1997; Brown *et al.*, 2000; Conant *et al.*, 1998; Howl *et al.*, 1998; Ohata *et al.*, 1997; Zhang *et al.*, 1997). Collectively, these characteristics make ECV304 cells particularly useful for experiments on astrocyte-endothelial calcium signaling mechanisms. The co-culture system does, however, not reproduce all the characteristics of the blood-brain barrier *in vivo*, and future work on more physiological preparations (e.g. brain slices) will be needed to confirm the present findings.

We used two different stimuli to invoke the two different calcium signaling pathways (intracellular $InsP_3$- and gap junction-dependent and extracellular purinergic) that are known to mediate intercellular calcium waves (Charles, 1998): increasing cytoplasmic $InsP_3$ by flash photolysis of caged $InsP_3$ to activate the intracellular gap junctional pathway, and probing the apyrase inhibitable fraction of calcium waves triggered by mechanical cell stimulation. The experiments with fast superfusion, cell-free gaps, apyrase and suramin clearly demonstrate that flash photolysis of caged $InsP_3$ invokes the gap junctional pathway in isolation without the contribution of an extracellulary released messenger liberated by $InsP_3$ or its consequential increase in cytoplasmic calcium. The peak calcium change attained with flash photolysis is, judged from the relative fluorescence change, in the order of 100-200 nM, and this is presumably not sufficient to trigger the release of an extracellular messenger. Previous work with flash photolysis of caged calcium has demonstrated that increasing cytoplasmic calcium to the level attained with flash photolysis of caged $InsP_3$ does not produce intercellular calcium waves, indicating that calcium by itself is not the signaling molecule (Leybaert *et al.*, 1998).

Astrocytes displayed a greater degree of gap junctional coupling as compared to endothelial cells, as can be concluded from the various experiments probing cell coupling. The higher coupling degree is also apparent from the flash photolysis experiments that demonstrated larger and faster propagating calcium waves in astrocytes as compared to endothelial cells. The experiments with mechanical cell stimulation are consistent with these data: apyrase had clearly less effect on astrocytic as compared to endothelial calcium waves (reduction to ~1/3 and ~1/12 respectively). The gap junctional pathway is thus more important in astrocytes than in endothelial cells.

Application of the two stimulation modes to the co-cultures demonstrated that the two signaling pathways are also operative between astrocytes and endothelial cells. This was also apparent from the two endothelial calcium response patterns observed following mechanical stimulation of an astrocyte (Fig. 4.10): sequential cell activation that follows the shortest path from the stimulated cell is more compatible with the extracellular pathway while a tortuous path is more reminiscent of the gap junctional pathway.

$InsP_3$-triggered calcium signals in endothelial cells showed extensive propagation in astrocytes; by contrast, calcium signals triggered in astrocytes only extended to a single endothelial cell. A probable explanation for this difference may reside in differences in the degree of gap junctional coupling, the geometry of the two cell types and the efficiency of electroporation loading. First, astrocytes show better gap junctional coupling and thus carry calcium signals farther away. Second, endothelial cells are thicker than astrocytes, thus closer to the electroporation electrode and thus more efficiently loaded with caged $InsP_3$. Third, because of the better loading and a thicker cytoplasmic layer exposed to photolysis light, a larger absolute amount of $InsP_3$ will be released in endothelial cells.

The absence of astrocyte-endothelial dye coupling, the negative electron microscopic findings and the presence of electrical coupling suggests a low coupling degree between astrocytes and endothelial cells, as has been observed between astrocytes and oligodendrocytes in co-culture (Ransom *et al.*, 1990). The degree of astrocyte to endothelial coupling must be very low because the two cell types maintain significantly different resting membrane potentials (-57 versus -38 mV). The reason why $InsP_3$-triggered calcium signals are then able to pass between so poorly coupled cells is probably related to an amplification effect: small amounts of $InsP_3$ diffusing in from an adjacent cell through gap junctions activate a measurable cytoplasmic calcium elevation because the resulting small amount of released calcium is amplified through calcium-induced calcium release at the $InsP_3$ receptor (Berridge, 1993; Bezprozvanny *et al.*, 1995). Because of this amplification effect, our findings suggest that intercellular calcium signal propagation following flash photolysis of caged $InsP_3$ is a very sensitive method to detect gap junctional coupling between cells, with a sensitivity that is in the order of what is achieved with electrophysiological techniques.

The experiments with mechanical stimulation and apyrase revealed that, in addition to the gap junctional pathway, astrocyte-endothelial calcium signals can also be carried by an extracellular purinergic messenger. This is in agreement with the findings obtained in other cell systems (Charles, 1998; Grafstein *et al.*, 2000; Paemeleire *et al.*, 2000b; Scemes *et al.*, 2000). The peak calcium change observed in astrocytes following mechanical stimulation was, judged from the relative fluorescence changes, slightly larger than the one observed following flash photolysis, and this is probably the reason why the extracellular release of an extracellular messenger comes into play. As to the messengers involved, there is evidence that both ATP and glutamate can be released upon a calcium elevation in astrocytes or glial cells (Calegari *et al.*, 1999; Cotrina *et al.*, 1998a; Guthrie *et al.*, 1999; Innocenti *et al.*, 2000; Parpura *et al.*, 1994). It has, however, convincingly been demonstrated that glutamate is not the extracellular messenger of calcium waves in astrocytes (Charles *et al.*, 1991; Enkvist *et al.*, 1992; Hassinger *et al.*, 1996; Venance *et al.*, 1997). Glutamate can neither be the astrocyte-endothelial calcium messenger as neither the presently used ECV304 and BCECs, nor human cerebrovascular endothelial cells are responsive to this messenger (Howl *et al.*, 1998; Morley *et al.*, 1998; Paemeleire *et al.*, 1999a). Endothelial calcium responses to purinergic messengers are mediated by P_{2Y2} (or P_{2U}) and P_{2Y1} receptors both in ECV304 cells (Conant *et al.*, 1998; Howl *et al.*, 1998; Paemeleire *et al.*, 2000a) and in primary BCEC cultures (Albert *et al.*, 1997; Ohata *et al.*, 1997; Zhang *et al.*, 1997).

The astrocyte-endothelial signals reported in this study not only concern the described changes in cytoplasmic calcium, but also imply communicated changes in $InsP_3$ and diacylglycerol (DAG). $InsP_3$, calcium and DAG are all important intracellular messengers in endothelial cells (Joo, 1994; Joo, 1993b). In addition, these signals originate from caveolae where the major endothelial intracellular signaling machinery is concentrated (Isshiki *et al.*, 1999; Isshiki *et al.*, 1998). Thus changes in these messengers will be expected to influence many aspects of blood-brain barrier functioning and transport. Activation of the $InsP_3$-calcium pathway has been reported to affect blood-brain barrier permeability (Abbott, 2000; Abbott *et al.*, 1991; Olesen, 1989), endocytosis (Stanimirovic *et al.*, 1996), ion transport (O'Donnell, 1991; O'Donnell *et al.*, 1995) and glucose transport through the GLUT-1 transporter (Dominguez *et al.*, 1996; Mitani *et al.*, 1995; Mitani *et al.*, 1996). Recent work from our group has shown that ATP and also histamine (acting through the $InsP_3$-calcium pathway) can acutely stimulate endocytosis and glucose uptake in endothelial cells (Braet *et al.*, 2000b; De Corte *et al.*, 2000). Neuronal electric activity can initiate propagating calcium changes in astrocytes (Dani *et al.*, 1992; Murphy *et al.*, 1993b) and thus, in principle, also activate astrocyte-endothelial calcium signals. We hypothesize that brain activation elicits neuron-astrocyte-endothelial calcium signals that result in increased glucose uptake from the blood, by stimulation of GLUT-1 mediated glucose transport, and thus that these calcium signals are instrumental in the coupling of neuronal metabolism to the extraction of glucose from the blood.

chapter

5

Photoliberating inositol-1,4,5-trisphosphate triggers ATP release that is blocked by the connexin mimetic peptide gap 26

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Abstract

Calcium signals can be communicated between cells by the diffusion of a second messenger through gap junction channels or by the release of an extracellular paracrine messenger. We investigated the contribution of both pathways in endothelial cell lines, either by photolytically increasing the intracellular messengers $InsP_3$ or calcium and recording the resulting intercellular calcium wave or by measuring the released ATP with a luciferin/luciferase assay. Photoliberating $InsP_3$ in a single cell within a confluent culture triggered an intercellular calcium wave, which was inhibited by the gap junction blocker α-glycyrrhetinic acid (α-GA), the connexin mimetic peptide gap 26, the purinergic inhibitors suramin, PPADS and apyrase and by purinergic receptor desensitization. $InsP_3$-triggered calcium waves were able to cross cell-free zones. Photoliberating $InsP_3$ in multiple cells triggered the release ATP which was blocked by buffering intracellular calcium with BAPTA and by gap 26. Gap 26, however, did not inhibit the gap junctional coupling between the cells as measured by fluorescence recovery after photobleaching. Photoliberating calcium did not trigger intercellular calcium waves or ATP release. We conclude that $InsP_3$-triggered ATP release through connexin hemichannels contributes to intercellular propagation of calcium waves.

5.1. Introduction

Intercellular calcium waves, i.e. cell-to-cell propagating changes of intracellular free calcium, are an ubiquitous form of cell-cell communication observed in cell cultures, slices and retina (Harris-White *et al.*, 1998; Newman, 2001; Rottingen *et al.*, 2000). Intercellular calcium waves can be communicated by an intracellular pathway that involves the passage of a calcium mobilizing second messenger like inositol 1,4,5-trisphosphate ($InsP_3$) through gap junctions (Boitano *et al.*, 1992; Leybaert *et al.*, 1998; Paemeleire *et al.*, 2000b), or an extracellular pathway that involves the release of a purinergic messenger like ATP acting on P_{2Y} receptors in a paracrine way (Cotrina *et al.*, 1998a; Cotrina *et al.*, 2000; Guthrie *et al.*, 1999; Hassinger *et al.*, 1996; Osipchuk *et al.*, 1992; Paemeleire *et al.*, 2000a). The involvement of an extracellular purinergic messenger-dependent pathway has more directly been investigated using bioluminescent ATP measurements, demonstrating ATP release in response to mechanical stimulation (Guthrie *et al.*, 1999; Newman, 2001), electrical stimulation (Hassinger *et al.*, 1996), the application of UTP (Cotrina *et al.*, 1998a) or by lowering extracellular calcium (Arcuino *et al.*, 2002). We have previously demonstrated that increasing the $InsP_3$ concentration in a single cell, by photolytic cleavage of its caged form, is a potent stimulus to initiate intercellular calcium waves that, in astrocytes, appear to be mainly carried by the gap junctional pathway (Braet *et al.*, 2001; Leybaert *et al.*, 1998). The aim of the present work was to determine whether an intracellular elevation of $InsP_3$ or calcium is also able to incite the extracellular/purinergic pathway and thus trigger the release of ATP.

Purinergic messengers like ATP or ADP are since long known to be released by vesicular release from dense core vesicles in platelets upon activation (Holmsen, 1994), a process mediated by $InsP_3$ and calcium (Brass *et al.*, 1985). ATP is equally well known to be co-released from neurons together with other neurotransmitters like noradrenaline (Bodin *et al.*, 2001b; Burnstock, 1995). ATP release from endothelial cells in response to shear stress or hypotonic stress has recently been suggested to be vesicular in nature (Bodin *et al.*, 2001a; Koyama *et al.*, 2001). However, hypotonic stress-induced ATP release from endothelial and/or epithelial cells has also been reported to be mediated by other release pathways (Schwiebert, 2001) including anion channels (Sabirov *et al.*, 2001; Sauer *et al.*, 2000), the cystic fibrosis transmembrane conductance regulator (CFTR) (Braunstein *et al.*, 2001; Sprague *et al.*, 1998), the multidrug-resistance P-glycoprotein (Abraham *et al.*, 1997) or, most recently, by means of so called connexin hemichannels, i.e. channels composed of six connexin subunits that do not connect to their counterparts in adjacent cells (Arcuino *et al.*, 2002; Cotrina *et al.*, 1998a; Cotrina *et al.*, 2000; Romanello *et al.*, 2001; Stout *et al.*, 2002).

In the present work we investigated the ability of $InsP_3$ and calcium to trigger purinergic messenger release in several cell lines of endothelial origin using photoliberation of these two messengers.

Our experiments, comprising both studies on calcium wave propagation and direct measurements of released ATP, demonstrate that $InsP_3$ and its subsequent calcium increase are able to trigger the release of ATP that is largely inhibited by the connexin mimetic peptide gap 26. Connexin mimetic peptides interact with the extracellular loops of the connexin subunit and some of them, like gap 27 have been demonstrated to be effective blockers of gap junction channels and thus gap junctional coupling (Evans *et al.*, 2001). Interestingly, our results show that gap 26 does not influence gap junctional dye coupling but has a drastic inhibitory effect on ATP release, suggesting a role for connexin hemichannels in the release process.

5.2. Materials and methods

5.2.1. Cell cultures

In this study we used two rat brain endothelial cell lines: RBE4 (Roux *et al.*, 1994) (kind gift of Dr. F. Roux, Neurotech, Evry, France) and SV-ARBEC (kind gift of Dr. D. Stanimirovic, Institute for Biological Sciences, NRC-CNRC, Ottawa, Canada), and the cell line ECV304 (European Collection of Animal Cell Cultures, Salisbury, UK). ECV304 cells, originally identified as endothelial cells from human umbilical vein (Takahashi *et al.*, 1990), have now been shown to be epithelial bladder cancer cells (Dirks *et al.*, 1999). ECV304 cells are interesting in that they have a purinergic receptor pharmacology that is similar to primary brain capillary endothelial cells (Albert *et al.*, 1997; Brown *et al.*, 2000; Conant *et al.*, 1998; Howl *et al.*, 1998; Ohata *et al.*, 1997; Zhang *et al.*, 1997) and they develop tight junctions and a low permeability barrier in co-culture with glial cells making them useful in blood-brain barrier models (Hurst *et al.*, 1996; Ramsohoye *et al.*, 1998). Clone 9 cells (European Collection of Animal Cell Cultures, Salisbury, UK) contain connexin-43 as the major connexin (Berthoud *et al.*, 1993) and were used as a positive control for the immunoblottings. Culture media (all from Gibco, Merelbeke, Belgium) were alpha-MEM/Ham's F10 (1:1) with 10 % fetal bovine serum (FBS), 1 µg/mL bFGF (Boehringer Mannheim, Brussels, Belgium) and 30 mg/mL geneticin (Gibco, Merelbeke, Belgium) for RBE4, Medium-199 with 10 % FBS, 100 mg/L peptone (Sigma, Bornem, Belgium), BME amino acids (Sigma, Bornem, Belgium) and BME vitamins (Gibco, Merelbeke, Belgium) for SV-ARBEC and Medium-199 with 2 mM glutamine and 10 % FBS for ECV304, and DMEM/Ham's F12 (1:1) with 2 mM glutamine and 10 % FBS for Clone 9. Cells were grown (except for immunoblotting) on glass bottom microwells (MatTek Corporation, Ashwood, MA, USA) coated with collagen type I (Boehringer Mannheim, Brussels, Belgium) and used for experiments upon confluency.

5.2.2. Agents

Fluo-3 acetoxymethyl ester (fluo-3 AM), D-myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (NPE-caged $InsP_3$), o-nitrophenyl EGTA acetoxymethyl ester (NP-EGTA-AM), BAPTA acetoxymethyl ester (BAPTA-AM), DMNB-caged fluorescein dextran (3000 MW), tetramethylrhodamine dextran (10000 MW) and 5-carboxyfluorescein diacetate (5-CFDA) were obtained from Molecular Probes (Leiden, The Netherlands). Probenecid, apyrase grade III, 18-α-glycyrrhetinic acid (α-GA), pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS) and adenosine 5' trisphosphate disodium salt (ATP) were from Sigma (Bornem, Belgium). Suramin hexasodium salt was from RBI (Natick, MA). The peptides gap 26

(VCYDKSFPISHVR) and des 5 (LEGHGDPLHLEEC) (Boitano *et al.*, 2000) were synthesized by the Biochemistry Department of Ghent University as a lyophilized compound at 95 % purity.

5.2.3. Western blotting

Proteins were extracted from cells by treatment with LDS buffer (Invitrogen, Groningen, The Netherlands) containing 50 µg/mL proteinase inhibitor cocktail (Sigma, Bornem, Belgium) and sonicated for two 10 second pulses. Total protein was determined with the BioRad (Nazareth, Belgium) DC protein assay. Proteins were separated on 10 % Bis Tris gels and transferred to nitrocellulose membranes. Blots were probed with polyclonal rabbit anti-rat connexin-43 antibody (Sigma, Bornem, Belgium) (1:10000) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma, Bornem, Belgium) (1:5000) and detection by the ECL chemiluminescent detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

5.2.4. Calcium imaging

Cytoplasmic free calcium was measured using the calcium-sensitive dye fluo-3 in combination with epifluorescence video microscopy and digital imaging. Cell cultures were loaded with fluo-3 for 1 hour at room temperature in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4) containing 10 µM fluo 3-AM (Molecular Probes, Leiden, The Netherlands) and 1 mM probenecid. Cultures were then washed with HBSS-HEPES and left at room temperature for 30 minutes for de-esterification. HBSS-HEPES was the bathing solution for all calcium imaging experiments Cells were viewed with an inverted epifluorescence microscope (Nikon Eclipse TE300, Analis, Ghent, Belgium) using a x40 oil immersion lens (CFI Plan Fluor, Nikon). Fluo-3 fluorescence images were obtained by excitation at 485 nm, reflection of a dichroic mirror with cut-off at 510 nm, and emission bandpass filtering at 535 nm (485DF22, 505DRLPXR and 535DF35 filters respectively from Omega Optical, Brattleboro, VT). Images were captured using an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) and stored on an S-VHS videorecorder (Panasonic, Avicom, De Pinte, Belgium) or directly to a PC equiped with an image acquisition and processing board (DT3155, Data Translation, Marlboro, MA). Each experiment was concluded by recording background images in a culture dish containing only medium and no cells; background images were subtracted from the fluo-3 fluorescence image sequences.

5.2.5. Caged compound loading, electroporation and UV spot illumination

The cells were loaded with caged $InsP_3$ by electroporation. Cultures were briefly rinsed with

electroporation buffer (300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1 mM $MgCl_2$ and 2 mM HEPES, pH 7.20) and thereafter a small volume (5 µl) of caged $InsP_3$ (200 µM) in electroporation buffer was added. This solution contained in addition dextran-rhodamine (100 µM) to visualise the electroporation zone. Electroporation was done with a parallel wire electrode (Pt-Ir, wire separation 0.8 mm, length 10 mm) positioned close to the cell surface (200 µm) and to which an oscillating high-frequency electric field was applied (20 repeats of ten 50 KHz bursts of 10 milliseconds duration at 400 V/cm field strength). The electroporation solution was then removed and the culture was washed three times with HBSS-HEPES. The zone of directly electroporated cells was, as estimated from the zone of dextran-rhodamine loaded cells, approximately 1 mm wide and 10 mm long. Caged $InsP_3$ (635 MW) can permeate through gap junctions and the distance to which significant caged $InsP_3$ spread occurred was determined by applying UV spot illumination (see below) at various distances away from the border of the dextran-rhodamine loaded zone and measuring the size of the calcium wave triggered in this way. The calcium wave size sharply decreased with the distance from the border of the dextran-rhodamine loaded zone and no waves could be triggered at approximately 150 µm away from the border. Caged $InsP_3$ thus spreads out from the electroporated zone, making the total zone of caged $InsP_3$ loading to be in the order of 1.3 mm by 10 mm.

Caged calcium loading was done by ester loading the cells with 5 µM NP-EGTA-AM for 10 minutes in 1 mL HBSS-HEPES containing probenecid (1 mM), followed by 30 minutes de-esterification (all at room temperature).

UV spot illumination for photorelease in calcium imaging experiments was performed with an Hg-arc lamp coupled to the microscope epifluorescence input through a dichroic mirror with cut-off at 400 nm (400DCLP02, Omega Optical, Brattleboro, VT) and focused to the field diaphragm plane by means of two plano-convex fused silica lenses (SPX046 and SPX049, Newport, Leuven, Belgium). The UV beam was bandpass filtered at 330 nm (330WB80, Omega Optical, Brattleboro, VT) and the exposure time (0.2 s) was controlled by a mechanical iris diaphragm shutter (Wollensak, Rochester, NY). The UV spot had a half-energy diameter of 10 µm, as determined by flashing a thin layer of DMNB-caged fluorescein dextran at 3 mM mixed (1:1) with Dako Glycergel (Dako Corporation, Carpinteria, CA). Regular inspection of the UV spot was done by illuminating a thin layer of Lucifer Yellow solution (as illustrated in Fig. 5.1H). To determine whether the UV spot only covered a single cell, we overloaded the cultures with NP-EGTA (10 µM NP-EGTA-AM for 60 minutes), resulting in heavy calcium buffering in all the cells loaded because of the high calcium affinity of NP-EGTA in its caged non-photolysed form. UV exposure liberates calcium only in the illuminated cell, while non-illuminated cells cannot display any propagated calcium signal because of effective buffering by non-photolysed NP-EGTA. Applying this kind of protocol showed that the photolytic UV light was only directed to a single target cell. Electroporation loading and flash

photolysis are described in further detail in Leybaert *et al.* (2001).

5.2.6. Cell-free zones and mechanical cell stimulation

Cell-free lanes were brought about by lineary scratching the cell cultures over approximately 10 mm with a thin syringe needle 4 hours before the actual experiment. The same procedure was used to elicit ATP release, as a positive control condition, in the experiments where ATP was measured (see below). Mechanical stimulation of a single cell was used in the experiments where the side effect of loading with NP-EGTA on cytoplasmic calcium buffering was tested. This was done by gently touching the cell with a glass micropipette (2 µm tip size) mounted on a micromanipulated piezoelectric device and driven to produce a short (100 milliseconds) tip displacement over approximately 5 µm.

5.2.7. Fluorescence recovery after photobleaching (FRAP)

Gap junctional coupling was investigated in ECV304 cells using the FRAP technique. Confluent monolayer cultures were loaded with the gap junction permeable fluorescent tracer 5-CFDA (25 µM) for 30 minutes at room temperature and kept 10 minutes in HBSS-HEPES without 5-CFDA. Cultures were then transferred to the microscope stage and fluorescence within a single cell was photobleached by spot exposure to 500 nm light (500DF50, Omega Optical, Brattleboro, VT) over a period of 10 s. The setup for spot illumination was the same as the one used for flash photolysis, except for the bandpass filter (500 instead of 330 nm) and a 50/50 beamsplitter (XF121, Omega Optical, Brattleboro, VT) replacing the dichroic. Recovery of fluorescence through dye influx from neighbouring cells was quantified at 9 minutes after the start of photobleaching. A correction was made for spontaneous bleaching that occurred because of continuous exposure to the 485 nm excitation light. This was done by recording the fluorescence profile in cells not exposed to the 500 nm spot bleach and correcting the recovery signal in proportion to the spontaneous fluorescence decay.

5.2.8. Extracellular ATP measurements

ATP release in response to photoliberation of $InsP_3$ or calcium was determined in ECV304 cultures using an ATP bioluminescent luciferin/luciferase assay kit (product no. FL-AA, Sigma, Bornem, Belgium). The photolytic UV light in these experiments was applied as a *field* illumination (not spot illumination) exposing multiple cells. To that purpose, light from an Hg-arc lamp with quartz collector lens was directed through a shutter, a 330 nm bandpass filter and a mirror (10D510AL.2, Newport, Leuven, Belgium) to form an image of the arc at the cell culture (covering an area of

approximately 6 by 8.0 mm). The total energy dose per unit of surface area applied in UV field illumination experiments was adjusted so that it was equal to the energy dose per unit of surface in the spot illumination experiments. This was done by adjusting the UV exposure time so that the fluorescence increase brought about by photoreleasing fluorescein from thin layers of caged fluorescein dextran (prepared as described above) was the same in both spot and field illumination setups. The UV exposure time for field illumination was 2 seconds determined in this way. The photolytic efficiency with this exposure time was in the order of the quantum efficiency of caged $InsP_3$ (0.65), indicating that all of the caged probe present in the cultures was effectively photocleaved upon a single field illumination exposure.

Immediately after UV exposure, 100 µL of 200 µL supernatant was collected from the cell cultures and transferred to 100 µL ATP assay mix solution (used at 5-fold dilution). Light emission from the 200 µL mix was then measured with a custom build luminometer consisting of a photomultiplier tube (9924B, Thorn-Emi Electron Tubes, Middlesex, UK) mounted in a light-tight housing with a sample holder. Luminescence was quantified as photon counts within a time window of 100 milliseconds and average photon count values were calculated from 100 repeated measurements on the same specimen (10 seconds measurement time per sample). Mechanical movement of the cell cultures during manipulation was strictly minimized as small shocks to the cultures or solution changes produced by themselves measurable ATP release (as reported by Abraham *et al.*, 1997. Calibration curves with ATP were performed in a concentration range of 50-1000 nM. The pharmacological agents used in the ATP measurement experiments were tested for side effects on the luciferin/luciferase assay, but none of them had any significant effect on the ATP-calibration curves.

To make the experimental conditions of the different cell culture groups comparable, all cultures received UV field illumination, which was applied just before collection of the supernatant. Baseline (non-triggered, spontaneous) ATP release was slightly but not significantly affected by exposure to the photolytic UV light (100 % without illumination versus 88 % $\pm$ 15 % with UV illumination; n=7; p=0.23). A group of cell cultures received a linear scratch (applied as described above), which served as a positive control condition because scratching the cultures liberates ATP from destroyed cells. The scratch was applied immediately after UV illumination and just before collection of supernatant. NP-EGTA loading and caged $InsP_3$ loading was done as described above (ester loading and electroporation loading respectively). Cell loading with the calcium buffer BAPTA was done before electroporation loading with caged $InsP_3$, by ester loading with 25 µM BAPTA-AM for 45 minutes in HBSS-HEPES containing probenecid (1 mM), followed by 30 minutes de-esterification (all at room temperature). Alpha-GA and gap 26 were applied after electroporation, for the time and concentrations indicated in the Results section, and were removed just before photolytic illumination.

It should be considered that in the ATP release experiments some photoliberated $InsP_3$ will diffuse, through gap junctions, to cells beyond the borders of the caged $InsP_3$ loaded zone, thereby extending the zone of cells potentially contributing to ATP release and introducing a gap junction-dependent component to the $InsP_3$-triggered ATP release. The extent of $InsP_3$ spread over the culture via gap junctions is expected to be comparable to the extent of caged $InsP_3$ spread (both have similar molecular weights, 437 and 635 respectively), which was determined to be in the order of 150 µm as described above. Such spread will occur at both lateral sides of the caged $InsP_3$ loaded zone (measuring 1.3 mm by 10 mm). The gap junction-dependent component in the $InsP_3$-triggered ATP release signal will derive from this diffusion zone and its relative contribution to the $InsP_3$-triggered ATP release signal was calculated to be in the order of 20-25 %. This means that the inhibitory effect of the gap junction blocker α-GA on $InsP_3$-triggered ATP release *per se* will be overestimated by a similar percentage.

5.2.9. Data analysis and statistics

Calcium changes are expressed as relative fluo-3 fluorescence changes, i.e. $\Delta F/F_0 = [F - F_0] / F_0$ with F_0 the fluorescence before stimulation and F the time-dependent fluorescence signal after stimulation.

The magnitude of $\Delta F/F_0$ changes triggered by photorelease of $InsP_3$ or calcium in the photostimulated cell was determined 2.5 seconds after the end of the illumination artefact. The extent of cell-to-cell propagation of calcium changes was determined from the size of the intercellular calcium wave at its maximal state of extension and was quantified by determining the surface area where $\Delta F/F_0$ was above a threshold of 50 %. Average $\Delta F/F_0$ changes for the whole calcium wave were calculated as the average of all above threshold pixel values.

The data are expressed as mean $\pm$ s.e.m. with n denoting the number of experiments. Statistical significance was tested using a t-test for unpaired observations with $p<0.05$. Multiple groups were compared using variance analysis and the Student-Newman-Keuls test.

5.3. Results

5.3.1. Photoreleasing $InsP_3$ triggers intercellular calcium waves

Photoreleasing $InsP_3$ in a single endothelial cell triggered a transient increase of cytoplasmic free calcium propagating to neighboring cells as an intercellular calcium wave (Fig. 5.1A-C). Application of the photolytic UV light to cultures not loaded with caged $InsP_3$ did not trigger any calcium change. At their maximal extent of propagation, the induced calcium waves had an above threshold ($\Delta F/F_0$>50 %) surface area of 4836 $\pm$ 409 µm² (n=50) for RBE4, 6982 $\pm$ 375 µm² (n=55) for SV-ARBEC and 7595 $\pm$ 341 µm² (n=29) for ECV304 (RBE4 wave size significantly smaller than SV-ARBEC and ECV304 wave sizes [p<0.0001]; SV-ARBEC not different from ECV304). There were no significant differences between the cell lines in the magnitude of the average calcium increase taken over all cells contributing to the calcium wave ($\Delta F/F_0$ averaged over all cells at maximal wave extension amounted to 111.0 % $\pm$ 2.0 % [n=134]; pooled data for all three cell lines).

$InsP_3$-triggered intercellular calcium waves were able to cross cell-free zones (19.0 $\pm$ 2.6 µm wide [n=10]) (Fig. 5.1D-G). This indicates that the wave propagation mechanism also contains an extracellular, non-gap junction dependent component, as has been demonstrated for mechanically triggered calcium waves (Cotrina *et al.*, 1998b; Guthrie *et al.*, 1999; Hassinger *et al.*, 1996; Paemeleire *et al.*, 2000a; Sauer *et al.*, 2000). We investigated the mechanisms of $InsP_3$-triggered intercellular calcium waves using pharmacological agents.

5.3.2. Alpha-GA and gap 26 reduce the size of $InsP_3$-triggered calcium waves

The contribution of the intracellular/gap junctional pathway to calcium signal propagation was studied with the gap junction blocker 18-α-glycyrrhetinic acid (α-GA) and the connexin mimetic peptide gap 26, a peptide that mimics a 13 amino acid sequence on the first extracellular loop of connexin-43 (Boitano *et al.*, 2000). Connexin-43 immunoreactivity was present in the three cell lines used as evidenced by Western blotting (Fig. 5.2).

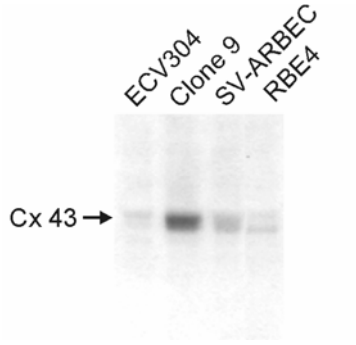

Fig. 5.2 *Immunoblotting of connexin-43 in the different cell lines used. The second lane (Clone 9; liver epithelial cells) represents the positive control condition. Connexin-43 expression could be detected in all the cell lines used, be it at lower levels as compared to Clone 9.*

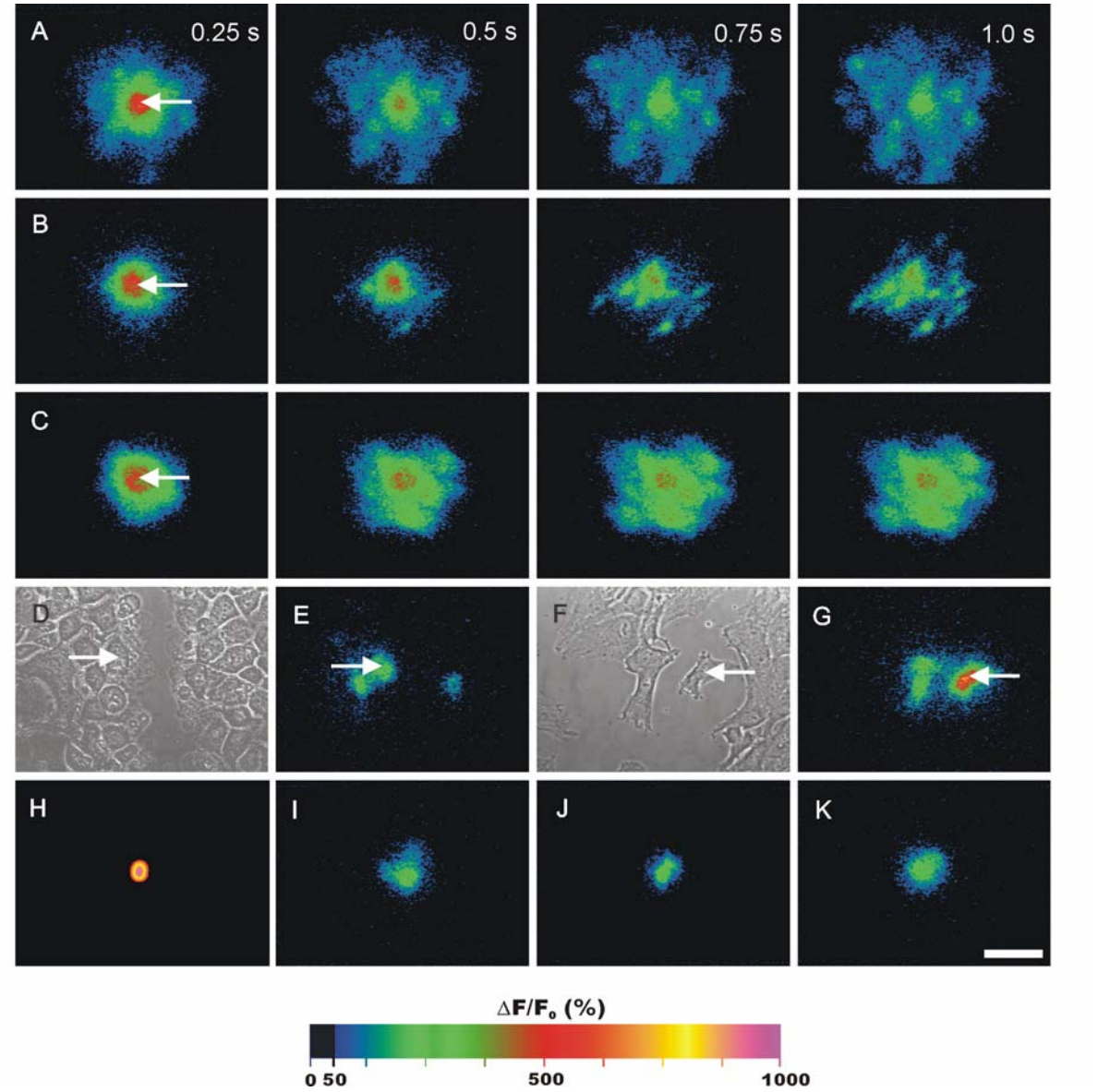

Fig. 5.1 *$InsP_3$-triggered cell-to-cell propagating calcium signals. A, B and C. Time sequences of intercellular calcium waves triggered by photoliberation of $InsP_3$ in confluent cultures of ECV304 (A), RBE4 (B) and SV-ARBEC (C). The images depict relative fluo-3 fluorescence changes ($\Delta F/F_0$) color coded as indicated in the color bar. The numbers in the right corner of each panel indicate the time after the start of photostimulation. The arrows indicate the position of the UV photostimulation spot. D-E and F-G. $InsP_3$-triggered calcium signals propagate across cell-free zones. Photoliberation of $InsP_3$ in a single cell (arrow) at the left side of the cell-free lane induced calcium changes in a cell at the opposite side of the lane (ECV304). Photoliberation of $InsP_3$ in a single isolated cell (arrow) in a subconfluent culture triggered calcium changes in a neighboring cell (SV-ARBEC). D and F are phase contrast images. H. Image of the photostimulation spot (half-energy diameter approx. 10 µm). I, J and K. Photolytic release of caged calcium (NP-EGTA) triggered a calcium increase in the stimulated cell but did not induce calcium changes in surrounding cells (ECV304 in I, RBE4 in J and SV-ARBEC in K). The calibration bar in panel K measures 50 µm and applies to all panels.*

Alpha-GA (50 µM, 30 minutes) reduced the wave size in the three cell lines (Fig. 5.3) but the effect was the most pronounced in RBE4 cells (approximately 60 % reduction). The effect of gap 26 (0.25 mg/mL, 30 minutes) more or less paralleled the effects of α-GA except in ECV304 where its inhibitory influence was stronger (approximately 60 % reduction) as compared to α-GA. The control peptide des 5, which mimics a 13 amino acid sequence on the intracellular and thus inaccessible loop of connexin-32 (Boitano *et al.*, 2000), had no effects on the size of intercellular calcium waves (not shown).

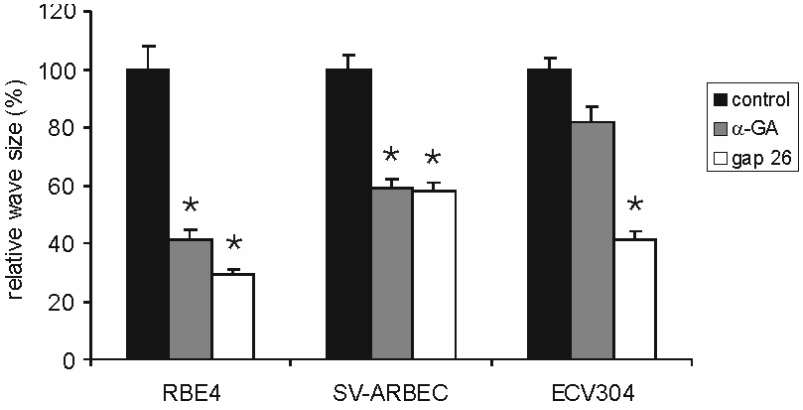

Fig. 5.3 *Effects of gap junction blockers on intercellular calcium wave propagation. Effects of the gap junction blocker α-glycyrrhetinic acid (α-GA; 50 µM, 30 minutes) and the connexin mimetic peptide gap 26 (0.25 mg/mL, 30 minutes) on the calcium wave size (surface area) expressed relative to the control condition. *significantly below control (p<0.01).*

Alpha-GA and gap 26 were tested for their effects on gap junctional coupling using the fluorescence recovery after photobleaching (FRAP) technique on 5-CFDA labeled ECV304 cells (Deleze *et al.*, 2001). Alpha-GA significantly reduced the gap junctional coupling between the cells, while gap 26 had no effects (Table 5.1). Prolonging the incubation period with gap 26 to 2 hours did neither inhibit the gap junctional coupling. The control peptide des 5 had no effects on coupling as expected (not given). In order to rule out the possibility that α-GA or gap 26 inhibited the extracellular/purinergic pathway, we checked whether these agents influenced calcium transients triggered by ATP superfusion (10 µM ATP, applied 1 minute). The two compounds had no significant effects on the peak amplitude of ATP–triggered calcium transients in ECV304 cells ($\Delta F/F_0$ = 138.4 ± 8.4 % in control, 120.0 ± 13.4 % with α-GA and 137.8 ± 12.2 % with gap 26; n=6 for each), indicating that these agents do not interfere with the cascade downstream from ATP release. The influence of these agents on the primary event in the extracellular/purinergic pathway, namely the release of ATP by the cells, was also studied and the results of these experiments are shown below.

Effect of gap junction and purinergic inhibitors on FRAP

Experimental condition	Fluorescence recovery (%) Mean ± SEM (n)
control	20 ± 5 (11)
α-GA	7 ± 2* (10)
gap 26	21 ± 3 (11)
suramin	17 ± 7 (11)
PPADS	18 ± 3 (8)
apyrase	26 ± 6 (8)

* significantly below control ($p<0.05$)

Table 5.1 *Effects of gap junction blockers and purinergic inhibitors on gap junctional communication studied with fluorescence recovery after photobleaching (FRAP). Alpha-GA significantly reduced fluorescence recovery, while gap 26 had no effect. Suramin, PPADS and apyrase did not significantly affect fluorescence recovery, indicating that these substances acting on the purinergic pathway have no side effects on the gap junctional pathway.*

5.3.3. Purinergic inhibitors also reduce the size of $InsP_3$-triggered intercellular calcium waves

The contribution of the extracellular/purinergic pathway was investigated with the purinergic receptor antagonists suramin (P_{2Y} and P_{2X} blocker) and PPADS (non-specific P_{2Y} blocker), the purine degrading enzyme apyrase and receptor desensitization with ATP. Suramin (100 µM, 10 minutes), PPADS (10 µM, 10 minutes), apyrase (40 IU/mL, 10 minutes) and ATP (10 µM, 10 minutes) all decreased the extent of wave propagation in the three cell lines but the efficacy of this effect varied between the cell lines used (Fig. 5.4). The effect was most pronounced in ECV304 cells (approximately 50 % reduction) and the least pronounced in RBE4 (approximately 25 % reduction). Exposing ECV304 cells to the combination of α-GA, PPADS and apyrase (concentrations and exposure time as mentioned higher) significantly reduced the calcium wave size to 34.9 ± 4.3 % of the 100 % control size (n= 29; p=0.0001; the remaining response comprised approximately 5 ± 1 cells, meaning that only the cells immediately adjacent to the stimulated cell contributed).

To rule out the possibility that some of the purinergic inhibitors acted directly on the intracellular calcium signaling cascade downstream from the purinergic receptor, we investigated whether these substances influenced calcium transients triggered by photorelease of $InsP_3$. None of the agents used had a significant effect on the peak amplitude of $InsP_3$–triggered calcium transients, indicating that these agents do not interfere with the cascade downstream from $InsP_3$-triggered store release (data not shown). We also determined whether the purinergic inhibitors influenced gap junctional coupling: suramin, PPADS and apyrase did not significantly affect the FRAP signal (Table 5.1), ruling out side effects of these purinergic inhibitors on gap junction channels.

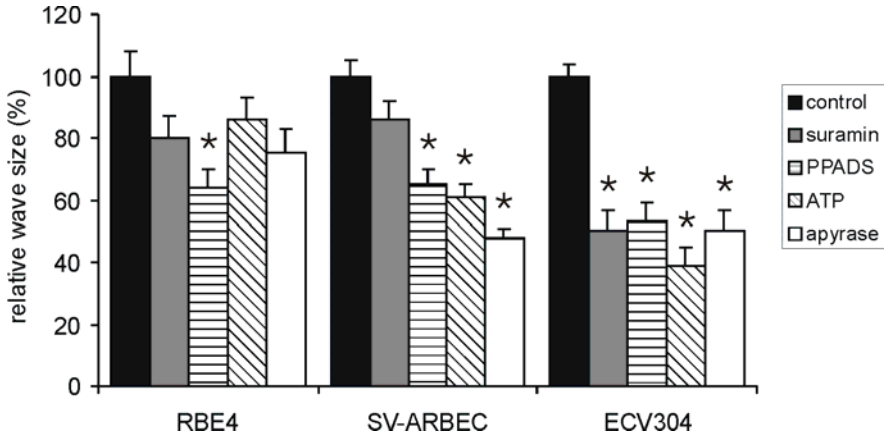

Fig. 5.4 *Effects of purinergic inhibitors on intercellular calcium wave propagation. Effects on the calcium wave size of the purinergic receptor antagonists suramin (100 µM, 10 minutes) and PPADS (10 µM, 10 minutes), of receptor desensitization with ATP (10 µM, 10 minutes) and of ATP/ADP degradation by the enzyme apyrase (40 IU/mL, 10 minutes). *significantly below control (p<0.01).*

5.3.4. Photoreleasing calcium does not trigger intercellular calcium waves

Photoreleasing calcium ions in a single cell, using the caged calcium compound NP-EGTA (5 µM AM ester, 10 minutes loading time), triggered an increase of intracellular free calcium in the photostimulated cell but did not trigger any calcium signal in neighboring cells (Fig. 5.1I-K). The magnitude of the induced calcium increase in these experiments was not different from the calcium increase triggered by photoreleasing $InsP_3$ ($\Delta F/F_0$ = 196.8 % ± 7.2 % [n=71] for photorelease of calcium versus 184.8 % ± 7.6 % [n=84] for photoliberation of $InsP_3$; pooled data from the 3 cell lines). Subsequent mechanical stimulation (with a micropipette) of the photostimulated cell always triggered an intercellular calcium wave, excluding the possibility that NP-EGTA in its caged, non-UV exposed and hence high calcium-affinity form, substantially buffered cytoplasmic calcium. A separate series of experiments confirmed that the size of calcium waves triggered by mechanical cell stimulation was not significantly influenced by the short protocol of NP-EGTA loading used in these experiments (control size 19920 ± 824 µm² [n=25] versus 18524 ± 967 µm² [n=19] following NP-EGTA loading; pooled data from the 3 cell lines).

5.3.5. Photoreleasing $InsP_3$ triggers ATP release that is blocked by calcium buffering, α-GA and gap 26

The role of $InsP_3$ and calcium in triggering the release of a purinergic messenger was further investigated in experiments directly measuring the release of ATP in the extracellular medium in response to photolytic liberation of these two messengers using a bioluminescent ATP assay. The experiments were performed on ECV304 cells because calcium waves in these cells were the most affected by purinergic inhibition (see Fig. 5.4). Baseline ATP release in the cells amounted to 12,3 ± 1,2 pmoles per 100 µl (n=16), referred to as 100 %. Scratching the cultures with a needle (MS; positive control condition) produced ATP release that was significantly above the baseline recorded in control cultures (Fig. 5.5). Photolytically increasing calcium did not produce any significant ATP release in comparison to baseline. Increasing the amount of photolytically liberated calcium by increasing the NP-EGTA concentration and loading time (10 µM concentration and 60 minutes loading time as compared to 5 µM and 10 minutes loading time in the standard protocol) was neither effective in triggering any ATP release (data not shown). By contrast, photoliberating $InsP_3$ produced a large ATP signal (EP_{InsP_3}), significantly above the electroporated baseline ($EP_{baseline}$). Preventing the $InsP_3$-triggered calcium increase by ester loading the cells with the calcium chelator BAPTA reduced the $InsP_3$-triggered ATP release back to the baseline level. Incubation of the cells with α-GA or gap 26 (50 µM and 0.25 mg/mL respectively, 30 minutes) completely abolished the $InsP_3$-triggered ATP response and reduced the ATP release below the baseline level, indicating that the baseline ATP release is also affected. In a separate series of experiments α-GA and gap 26 reduced the basal non-stimulated ATP release to 55.7 % $\pm$ 22.9 % (n=5) and 39.8 % $\pm$ 6.8 % (n=4) of the baseline level respectively ($p<0.001$ for the gap 26 effect). Alpha-GA and gap 26 had no influence on the bioluminescent ATP assay per se.

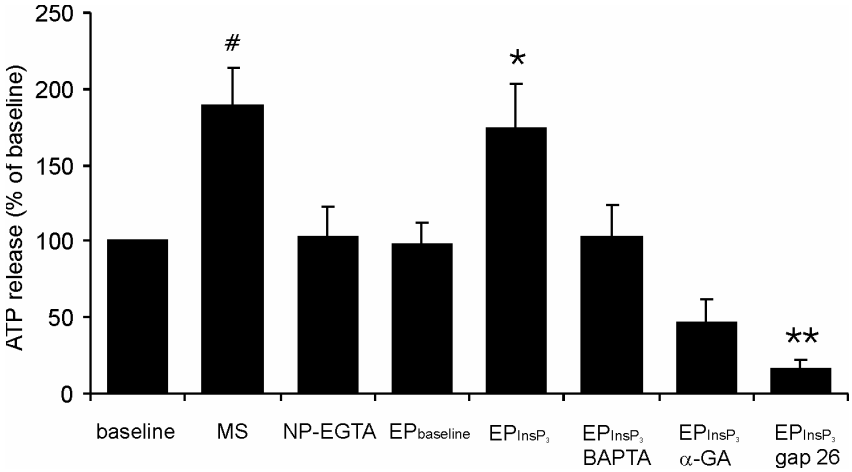

Fig. 5.5 *$InsP_3$ triggers ATP release in luciferin/luciferase bioassay experiments. Control cell cultures (baseline) produced baseline (spontaneous) ATP release. Mechanically stimulating the cultures (MS) by applying a linear scratch with a needle to release intracellular ATP, produced additional ATP release that was significantly above the baseline level. Cultures loaded with caged calcium (NP-EGTA) or cultures loaded by electroporation with vehicle ($EP_{baseline}$) showed ATP release upon photolytic stimulation that was not different from the baseline ATP release. By contrast, cultures loaded by electroporation with caged $InsP_3$ (EP_{InsP_3}) produced significantly increased ATP release following photolysis. The net component of $InsP_3$-triggered ATP release can be estimated from the difference between EP_{InsP_3} and $EP_{baseline}$. The $InsP_3$-triggered ATP release was abolished (ATP signal back to baseline level) by loading the cells with the calcium buffer BAPTA before photolysis (EP_{InsP_3} BAPTA). Interestingly, the $InsP_3$-triggered ATP release was also reduced by the connexin mimetic peptide gap 26 (EP_{InsP_3} gap 26) and the gap junction blocker α-GA (EP_{InsP_3} α-GA). The ATP signal in the presence of gap 26 or α-GA dropped below the baseline ATP level, and a separate series of experiments demonstrated that gap 26 significantly reduced the baseline component of ATP release (see text). #significantly above baseline ($p<0.05$), *significantly above $EP_{baseline}$ ($p<0.05$), **significantly below EP_{InsP_3} ($p<0.01$).*

5.4. Discussion

The present work was undertaken to investigate the pathways of calcium signal communication in response to elevations in cytoplasmic $InsP_3$ or calcium, and to determine whether such stimulation is able to trigger purinergic messenger release. The main finding of these experiments is that photoliberating $InsP_3$ triggers intercellular calcium waves that, in addition to the known involvement of the intracellular/gap junctional pathway, also invoke the extracellular/purinergic pathway. Increasing $InsP_3$ was demonstrated to directly trigger the release of ATP, and this stimulated release was dependent on the downstream calcium increase and was largely suppressed by the gap junction blocker α-GA and the connexin mimetic peptide gap 26.

Intercellular calcium waves were triggered by spot release of $InsP_3$ or calcium ions using flash photolysis, a technique we have used in previous work (Braet *et al.*, 2001; Leybaert *et al.*, 1998; Leybaert *et al.*, 2001). $InsP_3$-triggered intercellular calcium waves differed in size between the different cell lines, with ECV304 displaying the largest and RBE4 the smallest extent of wave propagation. The contribution of the two signaling pathways that mediate wave propagation was also different: the intracellular/gap junctional pathway was, judged from the effects of the gap junction blocker α-GA, the most pronounced in RBE4 while the extracellular/purinergic pathway appeared to be the most pronounced in ECV304 (Fig. 5.3 and 5.4). Exposure of ECV304 cells to a cocktail consisting of purinergic blockers (PPADS and apyrase) and α-GA demonstrated an additive effect compatible with two separately acting signaling pathways: α-GA alone removed 18 % of the calcium wave size, the purinergic inhibitors removed another 50 % (giving a summed inhibitory effect of 68 %) and the cocktail accordingly reduced the calcium wave size over approximately 65 %. Because the contribution of the extracellular purinergic pathway was so clear in ECV304 cells, we used these cells to investigate whether ATP release could be demonstrated more directly by using the luciferin/luciferase bioluminescent ATP assay. Our experiments clearly demonstrate that photoliberation of $InsP_3$ is sufficient to trigger ATP release that, estimated from the ATP assay calibration curves, attained a magnitude in the order of 200 nM in the 200 µl solution bathing the cells. Local concentrations close to the plasma membrane will be expected to be much higher immediately following release and before diffusion distributes the liberated ATP over the extracellular solution (Newman, 2001).

The most notable results in this study derive from the effect of the connexin mimetic peptide gap 26. Connexin mimetic peptides are short synthetic peptides that mimic a sequence on one of the extracellular loops of the connexin subunit (Evans *et al.*, 2001). The most frequently used peptide is gap 27 which mimics a highly conserved SRPTEK sequence present in the second extracellular loop and which has been demonstrated to inhibit gap junctional coupling (Berman *et al.*, 2002;

Chaytor *et al.*, 1998). Gap 26 is a tridecapeptide (13 amino acids) composed of residue numbers 63-75 of the first extracellular loop of connexin-43, containing the SHVR amino acid motif (Rozental *et al.*, 2001b). We used gap 26 in our experiments because this peptide has been reported to be an effective inhibitor of mechanically triggered intercellular calcium waves in airway epithelial cells (Boitano *et al.*, 2000). Gap 26 did indeed significantly inhibit our $InsP_3$-triggered intercellular calcium waves, but it did not have any effect on dye coupling through junctional channels as evidenced by the FRAP experiments, despite the fact that connexin-43 was present in the cell lines used. Long incubations (2 hours) with the peptide were neither effective in blocking gap junction channels. Connexin mimetic peptides are hypothesized to prevent the docking of two hemichannels on adjacent cells, thereby preventing the formation of a functional gap junction channel between the two cells (Berthoud *et al.*, 2000). Presumably, the connexin turnover time in our conditions is so slow that still longer incubations are needed to reveal the channel blocking effect. The most remarkable finding in our experiments was, however, that the peptide produced a complete inhibition of $InsP_3$-stimulated ATP release and also strongly inhibited the basal non-triggered ATP release. These findings thus indicate that the inhibitory effect of gap 26 on the $InsP_3$-triggered calcium waves is not the result of decreased gap junctional coupling, but of a selective inhibition of the ATP release pathway. Effects of gap 26 downstream the paracrine ATP signaling cascade, i.e. at the level of the purinergic receptors or on intracellular signal transduction were excluded in appropriate control experiments.

Photoreleasing calcium from NP-EGTA in a single cell, did not trigger any propagating calcium changes in nearby cells, although the calcium concentration in these experiments was increased to the same level as obtained following photoliberation of $InsP_3$. This is in line with previous observations made in astrocytes (Fry *et al.*, 2001; Leybaert *et al.*, 1998), where it was concluded that calcium by itself is (at least in those cells) not the messenger that communicates calcium signals via the intracellular/gap junctional pathway because of slower cytoplasmic diffusion as compared to $InsP_3$ (Allbritton *et al.*, 1992). The present work indicates that photolytically increasing calcium is neither sufficient to invoke the extracellular/purinergic pathway of calcium signal propagation. This is confirmed by the bioluminescence experiments that showed no ATP response upon photoliberation of calcium. A similar conclusion has been reported by Wang *et al.* (2000) based on direct imaging of extracellular ATP concentration and mechanically stimulated calcium waves. Calcium is however necessary as our experiments demonstrate that $InsP_3$-triggered ATP release was largely inhibited by buffering intracellular calcium with BAPTA (Fig. 5.5). Most probably, the $InsP_3$-triggered calcium release from the stores occurs focally and in close association to the calcium-sensitive release machinery (Montero *et al.*, 2001; Petersen *et al.*, 1999), while photoreleasing calcium from its caged precursor brings about a global calcium elevation in the cytoplasm that is insufficient to reach concentrations high enough at the release site (Etter *et al.*, 1996). Consistent with this picture are the reports that calcium entry from the

extracellular space, which occurs in close proximity to the target release site, is an effective stimulus of ATP release in epithelial and endothelial cells (Hashimoto *et al.*, 2001; Mitchell *et al.*, 1998).

A consequence of the finding that increasing $InsP_3$ triggers the release of ATP is that this can contribute to regenerative calcium wave propagation. In general, regenerative calcium wave propagation can occur by three distinct mechanisms: first, calcium ions diffusing through gap junctions might trigger calcium-induced calcium release (Taylor, 1998), thereby regenerating a calcium signal in adjacent cells (D'Andrea *et al.*, 1997; Yule *et al.*, 1996; Zimmermann *et al.*, 1999). As argued in the previous paragraph, this kind of regeneration does not occur in our experimental conditions and in the endothelial cells used. Second, a cytoplasmic calcium increase might activate PLC (Pawelczyk *et al.*, 1998; Rebecchi *et al.*, 2000), thereby regenerating $InsP_3$ that diffuses to adjacent cells via gap junctions (Venance *et al.*, 1997). Third, $InsP_3$ and its subsequent calcium increase might release a paracrine messenger that acts on metabotropic receptors of adjacent cells, thereby activating PLC and regenerating $InsP_3$ (Hassinger *et al.*, 1996). Our present results indicate that this last pathway contributes to regenerative calcium wave propagation in the cell lines used.

Our experiments indicate that gap 26, at the concentration and incubation time used, is an interesting tool to dissect the pathways of intercellular calcium signal communication, as it selectively suppresses the ATP release pathway without affecting the gap junction channel conduit. This effect is likely to be mediated by specific interactions of the peptide with the first extracellular loop of connexin-43, as the control peptide des 5 had no effects. ATP release thus appears to be connexin-related, but the exact nature of this relation is not clear at present. Connexin hemichannels have been implicated in the release of nucleotides by the cell, and several authors have recently given experimental evidence for the involvement of such release pathway that is activated by lowering extracellular calcium (Arcuino *et al.*, 2002; Cotrina *et al.*, 1998a; Noma *et al.*, 1987; Pfahnl *et al.*, 1999; Romanello *et al.*, 2001; Stout *et al.*, 2002). Our present work suggests that an increase of intracellular $InsP_3$ and calcium might transduce to connexin-related ATP release, but this notion is difficult to combine with the well documented evidence that an increased cytoplasmic calcium concentration, with or without the help of downstream calcium-dependent protein kinases, results in inhibition of gap junction channels or hemichannels (Enkvist *et al.*, 1992; Lazrak *et al.*, 1993; Saez *et al.*, 1993). However, one aspect that should be taken into account is that channel closure only occurs after prolonged (minutes) exposure to the increased calcium condition while short lasting calcium transients (several tens of seconds), e.g. as occurs in the course of an intercellulary propagating calcium wave, are ineffective in blocking the channel (Giaume *et al.*, 1998). A point noteworthy to mention is that the first extracellular loop on the connexin-subunit, which contains the gap 26 mimetic motif, is part of a sequence involved in

voltage gating (Harris, 2001). Interaction of gap 26 with this loop, which on connexin hemichannels is not occupied by an apposed hemichannel and hence freely accessible, can thus be hypothesized to alter the gating properties of connexin hemichannels. Further work will be needed in order to characterize the nature of $InsP_3$-triggered and gap 26-inhibitable ATP release and its possible relation to other candidate ATP release pathways such as vesicular, CFTR-related, P-glycoprotein related or anion channel mediated release.

chapter

6

Pharmacological sensitivity of ATP release triggered by photoliberation of inositol-1,4,5-trisphosphate and zero extracellular calcium in brain endothelial cells

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Text and illustrations modidfied from the published version.

Abstract

Recently, ATP has gained much interest as an extracellular messenger involved in the communication of calcium signals between cells. The mechanism of ATP release is, however, still a matter of debate. In the present study we investigated the possible contribution of connexin hemichannels or ion channels in the release of ATP in GP8, a rat brain endothelial cell line. Release of ATP was triggered by photoactivation of $InsP_3$ or by reducing the extracellular calcium concentration. Both trigger protocols induced ATP release significantly above baseline. $InsP_3$-triggered ATP release was completely blocked by α–glycyrrhetinic acid (α–GA), the connexin mimetic peptides gap 26 and 27, and the trivalent ions gadolinium and lanthanum. ATP release triggered by zero calcium was, in addition to these substances, also blocked by flufenamic acid (FFA), niflumic acid and NPPB. Gap 27 selectively blocked zero calcium-triggered ATP release in connexin-43 transfected HeLa cells, while having no effect in wild-type and connexin-32 transfected cells. Of all the agents used, only α–GA, FFA and NPPB significantly reduced gap junctional coupling. In conclusion, $InsP_3$ and zero calcium-triggered ATP release show major similarities but also some differences in their sensitivity to the agents applied. It is suggested that both stimuli trigger ATP release through the same mechanism, which is connexin-dependent, permeable in both directions, potently blocked by connexin mimetic peptides, and consistent with the opening of connexin hemichannels.

6.1. Introduction

Cell-cell communication is a prerequisite for the normal functioning of many tissue types and is of utmost importance in the brain. The energy molecule ATP has a prominent role in intercellular signaling as it is constitutes an important first messenger of the ubiquitous paracrine purinergic signaling pathway (Bodin *et al.*, 2001a). Paracrine and autocrine ATP signaling have been implicated in various cell functions such as neurotransmission (Burnstock, 1995), smooth muscle contraction (Bergner *et al.*, 2002), inflammation (Bodin *et al.*, 1998) and cell volume regulation (Wang *et al.*, 1996). In addition, paracrine ATP signaling is a major component of calcium signal communication between cells which has been demonstrated in a myriad of cell types including glial cells (Scemes *et al.*, 2000) and endothelial cells (Moerenhout *et al.*, 2001). This pathway is also operative at the blood-brain barrier, carrying calcium signals between astrocytes and endothelial cells (Braet *et al.*, 2001). Several experimental protocols have been used to trigger the release of ATP from cells such as mechanical strain (Cotrina *et al.*, 1998b; Sprague *et al.*, 1998; Stout *et al.*, 2002; Wang *et al.*, 2000), hypotonic swelling (Jans *et al.*, 2002; Koyama *et al.*, 2001; Wang *et al.*, 1996), and inositol trisphosphate ($InsP_3$)-generating peptides such as angiotensin II, bradykinin and substance P (Katsuragi *et al.*, 2002; Tamesue *et al.*, 1998). As ATP is a highly charged molecule that cannot cross cell membranes by simple diffusion, several specific release mechanisms have been proposed. Vesicular ATP release, in co-release with other neurotransmitters is well known in neurons, and has also been reported in astrocytes (Bal-Price *et al.*, 2002; Coco *et al.*, 2003) and endothelial cells (Bodin *et al.*, 2001a). Other suggested pathways include anion channels (Sabirov *et al.*, 2001), ATP-binding cassette transporters (Abraham *et al.*, 1993), the CFTR transporter (Reisin *et al.*, 1994) and connexin hemichannels (Stout *et al.*, 2002), which are half of gap junction channels not connected to their counterparts on neighboring cells.

Functional connexin hemichannels were first reported in horizontal cells of the retina (DeVries *et al.*, 1992; Malchow *et al.*, 1993) and have since then been found in various native and transfected cell types (Ebihara *et al.*, 1993; John *et al.*, 1999a; Li *et al.*, 1996; Pfahnl *et al.*, 1999). Hemichannels behave like large conductance ion channels that share properties with gap junction channels in terms of permeability and gating (DeVries *et al.*, 1992; Li *et al.*, 1996; Trexler *et al.*, 1996). Connexin hemichannels can be activated by exposure to calcium-free solutions (DeVries *et al.*, 1992; Li *et al.*, 1996), by membrane depolarization (Hofer *et al.*, 1998) or by metabolic inhibition (Contreras *et al.*, 2002; John *et al.*, 1999a).

The present study was performed to investigate the effect of various pharmacological agents that have been used in investigations of ATP release mechanisms, such as gap junction blockers, trivalent ions, fenamates, ion channel blockers, on ATP release in blood-brain barrier endothelial cells. Two different protocols were used to trigger ATP release: one in which intracellular $InsP_3$ was

directly increased by photoliberating it from a caged precursor using flash photolysis, and the other involving exposure of the cells to a low extracellular calcium solution (further called zero extracellular calcium). We included in the series of possible ATP release blocking agents two connexin mimetic peptides that have been proposed as gap junction channel blockers (Berman *et al.*, 2002; Chaytor *et al.*, 1998). Our data suggest, first, that some of the applied substances block ATP release triggered by $InsP_3$ and zero extracellular calcium, indicating similarities between the two release pathways activated, second, that the pharmacological sensitivity of zero calcium-triggered ATP release and zero calcium-triggered dye uptake is very similar, indicating that the activated pathway is shared and bidirectional, and third, that connexin mimetic peptides block this pathway in a connexin-specific way.

6.2. Materials and methods

6.2.1. Cell cultures

In this study we used a rat brain endothelial cell line GP8 (Kind gift of Dr. J. Greenwood, Dept. of Ophthalmology, University College of London, London, UK) (Greenwood *et al.*, 1996). The cell culture was maintained in alpha-MEM/Ham's F10 (1:1) with 10 % FBS, 1 µg/mL bFGF (Boehringer Mannheim, Brussels, Belgium) and 30 mg/mL geneticin (Gibco, Merelbeke, Belgium). GP8 cells were grown on glass bottom microwells (MatTek Corporation, Ashwood, MA, USA) coated with collagen type I (Boehringer Mannheim) and used for experiments upon confluency. The HeLa parental and Cx43- and Cx32-transfected HeLa were grown in MEM with 10 % FBS, and 1 µg/mL puromycin was included in the medium for the transfected HeLa cells. HeLa cells were grown on 4-well plates (Nalge Nunc, Rochester, NY, USA).

6.2.2. Agents

$P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (further called caged $InsP_3$), 5-carboxyfluorescein diacetate (5-CFDA) , dextran tetramethylrhodamine (MW 10000) and dextran fluorescein (MW 70000) were obtained from Molecular Probes (Leiden, The Netherlands). The connexin mimetic peptides gap 26 (VCYDKSFPISHVR), gap 27 (SRPTEKTIFII) and des 5 (LEGHGDPLHLEEC) were synthesized by solid phase chemistry and purified by HPLC and purity (95 %) assessed by HPLC. Alfa-glycyrrhetinic acid, $GdCl_3$, $LaCl_3$, niflumic acid, flufenamic acid, glibenclamide, NPPB and quinine were purchased from Sigma (Bornem, Belgium).

6.2.3. Caged $InsP_3$ loading and photoliberation of $InsP_3$

Cultures were loaded with caged $InsP_3$ by electroporation as described by Braet *et al.* (2003b). Briefly, the cells were rinsed once with a poorly conducting electroporation buffer. Thereafter, a small volume (5 µL) of caged $InsP_3$ (200 µM) dissolved in electroporation buffer was added to a parallel wire electrode positioned close (250 µm) to the cell surface; the small amount of loading solution was trapped in this way, by capillarity, between the electrode wires and the cells. The cell membranes were transiently permeabilized by applying a radio-frequency electric field to the electrode, allowing the entry of caged $InsP_3$ into the cells. The electroporation solution was then removed and the culture was washed three times with Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4). Photoactivation of the caged probe, resulting in the liberation of $InsP_3$ inside the cells, was performed by 2 seconds UV exposure of the entire glass

surface of the microwell dish, using a Hg-arc lamp, a condenser lens and a 330 nm bandpass filter.**Extracellular ATP measurements**

Cellular ATP release was determined by using an EDTA-containing luciferin/luciferase assay kit (product no. FL-AA, Sigma, Bornem, Belgium). ATP release was triggered by two different protocols: either by photoliberating $InsP_3$ inside the cells or by 2 minutes exposure of the cells to a zero extracellular calcium solution (calcium- and magnesium-free HBSS-HEPES containing 1mM EGTA). In both cases 100 µL of 200 µL supernatant was collected immediately at the end of the trigger protocol (lasting 2 s for the photolysis protocol and 2 min for the zero calcium protocol) and was transferred to 100 µL ATP assay mix solution (used at 5-fold dilution). The mixture was placed in a sample holder of a custom build luminometer and light emission was measured as photon counts. Calibration curves with ATP were performed in a concentration range of 50-1000 nM. To ensure that the results obtained with the bioluminescent luciferin/luciferase assay were not altered by the pharmacological agents applied in this study, ATP calibration curves were also acquired in the presence of the concerned agents. None of the agents used, including lanthanides (see further), had any significant effect on the ATP calibration curves. All pharmacological agents were applied for a period of 30 minutes unless otherwise stated and were also present in the trigger solutions. The composition of the zero calcium trigger solution was different when the lanthanides gadolinium and lanthanum were used, because EGTA has a high affinity for these ions thereby effectively complexing them. In that case it contained 100 µM EGTA, instead of the 1 mM normally used, and an increased lanthanide concentration (150 µM gadolinium and 200 µM lanthanum) to obtain free ion concentrations in the order of the concentrations used in non-EGTA containing solutions. Corresponding control experiments without lanthanides were also performed with this 100 µM EGTA trigger solution. Lanthanides had no effect on the ATP calibration curves because the assay kit used in our experiments contained EDTA to complex these ions (Boudreault *et al.*, 2002). Baseline release in zero calcium-triggered ATP release was determined by exposure to normal HBSS-HEPES solution and baseline release in $InsP_3$-triggered ATP release was performed with vehicle electroporated cells. With both trigger protocols, the baseline ATP concentrations released by the cells in 200 µL medium amounted to approximately 45.5 nM.

6.2.5. Dye influx

We performed dye influx experiments to assess whether the zero calcium trigger could, in addition to releasing intracellular ATP into the extracellular space, also induce the uptake into the cells of an extracellulary added fluorescent marker. Monolayer cultures were rinsed twice with calcium- and magnesium-free HBSS-HEPES containing 1 mM EGTA and were then incubated for 30 minutes with 2 mM propidium iodide added to the same solution. The zero calcium solution was

adapted, as described higher, when lanthanides were used. Vehicle experiments were performed with normal HBSS-HEPES solutions. Pictures were acquired with a x10 objective, TRITC epifluorescence settings and a cooled CCD camera (SensiCam, PCO, Kelheim, Germany). In each culture, 10 images were taken in which average dye uptake was determined. Propidium iodide positivity was quantified by calculating the number of pixels with a fluorescence intensity above a threshold value. Comparison between manually counted propidium iodide positive cells and supra-threshold pixels showed a linear relation between both parameters. The number of cells demonstrating zero calcium-triggered propidium iodide uptake amounted to 13.0 ± 0.9 % of the cells (n=10).

6.2.6. Fluorescence recovery after photobleaching (FRAP)

Gap junctional communication was investigated using FRAP. Confluent monolayer cultures were loaded with the gap junction permeable fluorescent tracer 5-CFDA (25 µM) for 30 minutes at room temperature and kept 10 minutes in HBSS-HEPES without 5-CFDA. A single cell in the culture was then photobleached by 10 seconds exposure to 500 nm light. Recovery of the fluorescence caused by dye influx through gap junctions connecting to neighbouring cells was quantified at 2 minutes after photobleaching.

6.2.7. Data analysis and statistics

The data are expressed as mean ± s.e.m. with n denoting the number of experiments. Statistical significance was tested using a t-test for unpaired observations with $p<0.05$. Multiple groups were compared using variance analysis followed by the Dunnett test for multiple comparisons to the control group.

6.3. Results

6.3.1. ATP release triggered by photoliberating $InsP_3$

Baseline ATP release in GP8 cells (a brain endothelial cell line) amounted to 5,0 ± 0,7 pmoles per 100 µl (n=14), referred to as 100 %. Increasing the intracellular $InsP_3$ concentration by flash photolysis of caged-$InsP_3$ in these cells triggered ATP release that was significantly above the baseline level (Fig. 6.1). This demonstrates the presence of a net component of $InsP_3$-triggered ATP release. Incubation of the cells with the gap junction blocker α-glycyrrhetinic acid (α-GA 50 µM, 30 min) and the connexin mimetic peptides gap 26 and gap 27 (0.25 mg/mL, 30 min) completely abolished the $InsP_3$-triggered ATP response (Fig. 6.1). Gap 26 and gap 27 both mimic a sequence on the extracellular loop of connexin-43; by contrast, des 5 mimics an intracellular loop sequence of connexin-32, and this control peptide did not affect the triggered ATP release. The trivalent cations gadolinium (Gd^{3+}) and lanthanum (La^{3+}) have been used to block connexin hemichannels (John *et al.*, 1999a; Vanoye *et al.*, 1999), which are half gap junction channels not connecting to apposed cells that have been implicated in ATP release (Braunstein *et al.*, 2001; Taylor *et al.*, 1998). Both trivalents (Gd^{3+} 30 µM, La^{3+} 50 µM, 30 min) efficiently blocked $InsP_3$-triggered ATP release (Fig. 6.1).

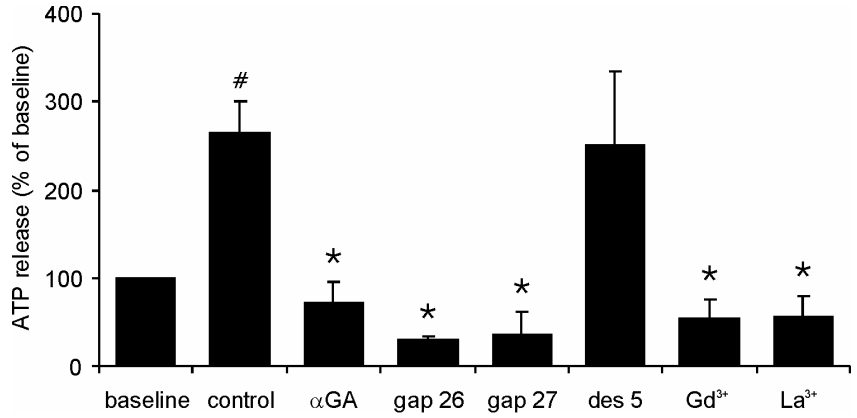

Fig. 6.1 *ATP release triggered by photoliberation of $InsP_3$ in GP8 brain capillary endothelial cells. ATP release levels were normalized to ATP release in vehicle electroporated cells (baseline). UV illumination of caged $InsP_3$ electroporated cells (control) triggered ATP release that was significantly above baseline. The induced release was inhibited by 30 minutes pretreatment of the cells with α-GA, gap 26, gap 27, Gd^{3+} and La^{3+}. Treatment with the control peptide des 5 had no effect on $InsP_3$-triggered ATP release. #p<0.005, significantly different from baseline; *p<0.05, significantly different from control with n at least 5.*

α-GA, the peptides and the trivalent reduced ATP release below baseline level, indicating that the baseline release is also affected. In a separate series of experiments α-GA, gap 26, gadolinium en

lanthanum reduced basal non-stimulated ATP to 68.5 % ± 12.8 % (n=4), 22.8 % ± 10.6 % (n=2), 55.2 % ± 6.4 % (n=3) and 19.0 % ± 3.1 % (n=3) respectively ($p<0.05$).

The fenamates flufenamic acid (FFA) and niflumic acid (NFA) have been reported to block hemichannel currents (Zhang *et al.*, 1998) and have also been used as gap junction blockers (Harks *et al.*, 2001). These substances (both at 100 µM, 30 min) did not inhibit $InsP_3$-triggered ATP release and NFA even tended to potentiate it (Fig. 6.2). Some ion channel blockers like NPPB and glibenclamide (Hashimoto *et al.*, 2001; Mitchell *et al.*, 1998) have been demonstrated to block ATP release while others such as quinine potentiated ATP release (Stout *et al.*, 2002). The chloride channel blocker NPPB and the potassium channel inhibitors glibenclamide and quinine (all at 100 µM, 30 min) did not influence $InsP_3$-triggered ATP release (Fig. 6.2).

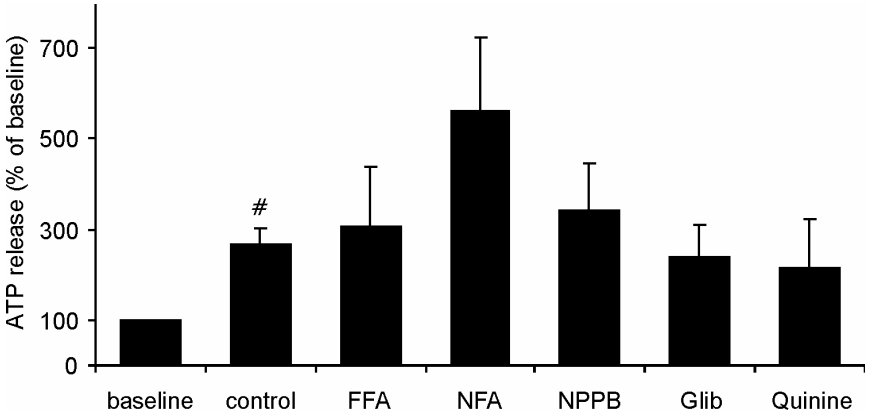

Fig. 6.2 *ATP release triggered by photoliberation of $InsP_3$ in GP8 brain capillary endothelial cells. FFA, NFA, NPPB, glibenclamide and quinine had no significant effect on $InsP_3$-triggered ATP release. #p<0.005, significantly different from baseline with n at least 5.*

6.3.2. ATP release triggered by zero extracellular calcium

ATP release by the cells has been suggested to be consistent with the opening of connexin hemichannels (Stout *et al.*, 2002), and exposure of the cells to zero extracellular calcium solutions has been used to trigger ATP release through this pathway (Arcuino *et al.*, 2002). We have applied the zero extracellular calcium trigger to investigate the effect of the inhibitory substances described above. Exposing GP8 cells to a zero extracellular calcium solution (1 mM EGTA, 2 min) triggered ATP release that was significantly above the baseline level. Zero calcium-triggered ATP release was significantly inhibited by α-GA, the connexin mimetic peptides gap 26 and gap 27, the trivalent ions (Fig. 6.3), the fenamates and NPPB (Fig. 6.4). The control peptide, glibenclamide and quinine had no effect on zero calcium-triggered ATP release.

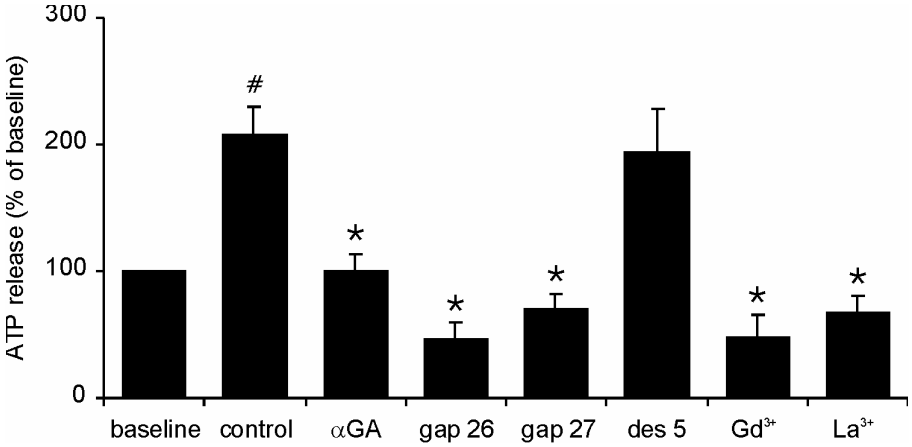

Fig. 6.3 *ATP release triggered by zero extracellular calcium. ATP release levels were normalized to ATP release in normal extracellular calcium conditions (baseline). Switching to a zero calcium buffer containing 1 mM EGTA (control) induced ATP release that was significantly above baseline. Pretreatment of the cells with α-GA, gap 26, gap 27, Gd^{3+} and La^{3+} significantly inhibited ATP release, while des 5 had no effect. #p<0.005, significantly different from baseline; *p<0.05, significantly different from control with n at least 5.*

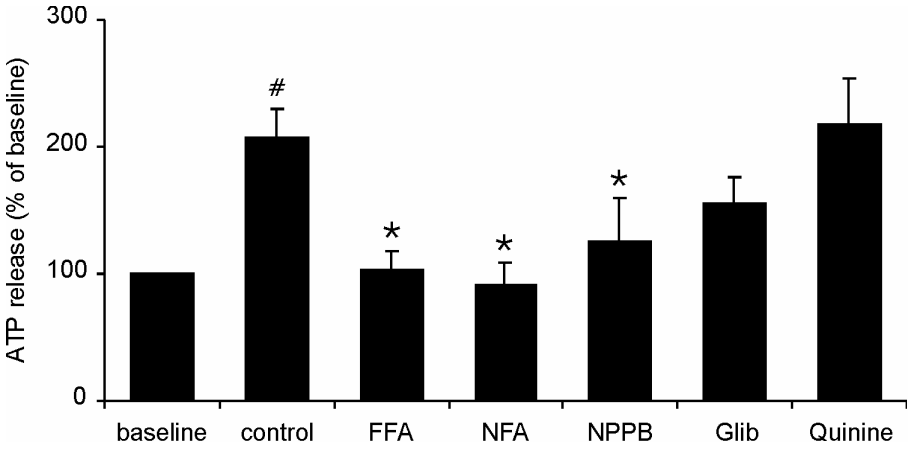

Fig. 6.4 *ATP release triggered by zero extracellular calcium. Zero calcium-triggered ATP release was significantly inhibited by FFA, NFA and NPPB. No effect could be observed with glibenclamide (Glib) or quinine. #p<0.005, significantly different from baseline; *p<0.05, significantly different from control with n at least 5.*

Gap 27 has been widely applied as a gap junction blocker (Chaytor *et al.*, 1999; De Vriese *et al.*, 2002; Kwak *et al.*, 1999) and we were therefore interested to further investigate the notable ATP release inhibiting action of this peptide on zero calcium-triggered ATP release in HeLa wild-type (HeLa WT) and connexin-43 transfected cells (HeLa Cx43) (Elfgang *et al.*, 1995). Zero calcium exposure triggered ATP release that was significantly above baseline in both HeLa WT and HeLa Cx43, but the release in HeLa Cx43 was much larger as compared to HeLa WT (Fig. 6.5). Gap 27 had no significant inhibitory effect on zero calcium-triggered ATP release in HeLa WT, but had a

strong and significant inhibitory effect in HeLa Cx43. HeLa cells transfected with connexin-32 (HeLa Cx32) displayed baseline and zero calcium-triggered ATP release similar to HeLa Cx43 but gap 27 had no inhibitory effect on ATP release in these cells, indicating that the action of gap 27 is connexin-selective (data not shown).

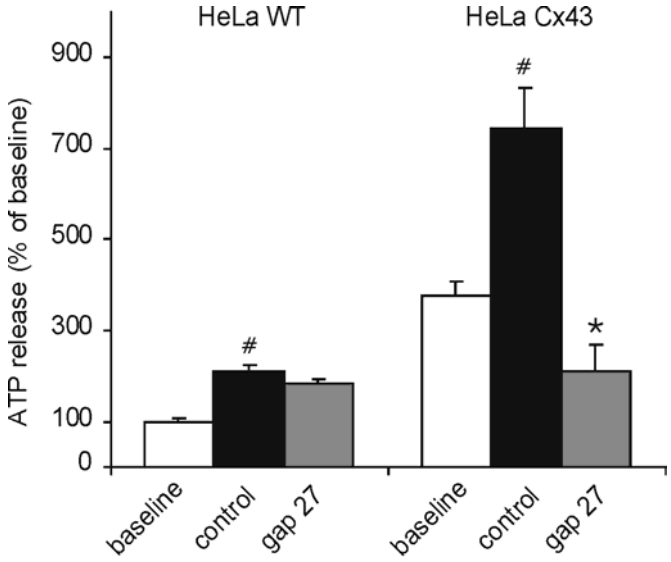

Fig. 6.5 *Effect of the connexin mimetic peptide gap 27 on triggered ATP release in connexin-transfected HeLa cells. Data are normalized to baseline release in parental HeLa cells. The baseline release in connexin-43 transfected HeLa cells was significantly above baseline release in parental cells. Both cell lines showed a significant increase of ATP release in response to zero calcium (control), but gap 27 only significantly depressed triggered ATP release in connexin-43 transfected cells. #p<0.005, significantly different from corresponding baseline value; *p<0.05, significantly below the control response; n at least 8.*

6.3.3. Dye influx triggered by zero extracellular calcium

Zero calcium conditions have been utilized to demonstrate the opening of plasma membrane channels, presumably connexin hemichannels, and the entry of fluorescent dyes into the cells (DeVries *et al.*, 1992; Li *et al.*, 1996). We have employed a similar protocol to investigate the effect of the various inhibitor substances applied in the previous series of experiments. Thirty minutes exposure to zero extracellular calcium triggered significant uptake into the cells of small dyes like propidium iodide (MW 562) and carboxyfluorescein (MW 376) while excluding larger dyes like dextran tetramethylrhodamine 10 kDa or dextran fluorescein 70 kDa. The zero calcium-triggered propidium iodide dye influx was temperature dependent and was characterized by a Q_{10} value (increase in uptake for a 10 °C temperature change) of 1.30 ± 0.16 (n=6); baseline dye influx had a Q_{10} in the same order: 1.42 ± 0.06 (n=6). These values are compatible with propidium iodide being transported into the cells by free diffusion through large conductance channels. The zero calcium-triggered propidium iodide influx displayed a response pattern to the various inhibitor substances

that was very similar to the response pattern observed for zero calcium-triggered ATP release: α–GA, gap 26, gap 27, gadolinium, lanthanum, FFA and NFA all significantly inhibited dye influx. NPPB decreased it to a lesser extent, and des 5 and glibenclamide had no inhibitory effects (Fig. 6.6). By contrast, quinine which did not influence triggered ATP release, did inhibit zero calcium-triggered dye influx.

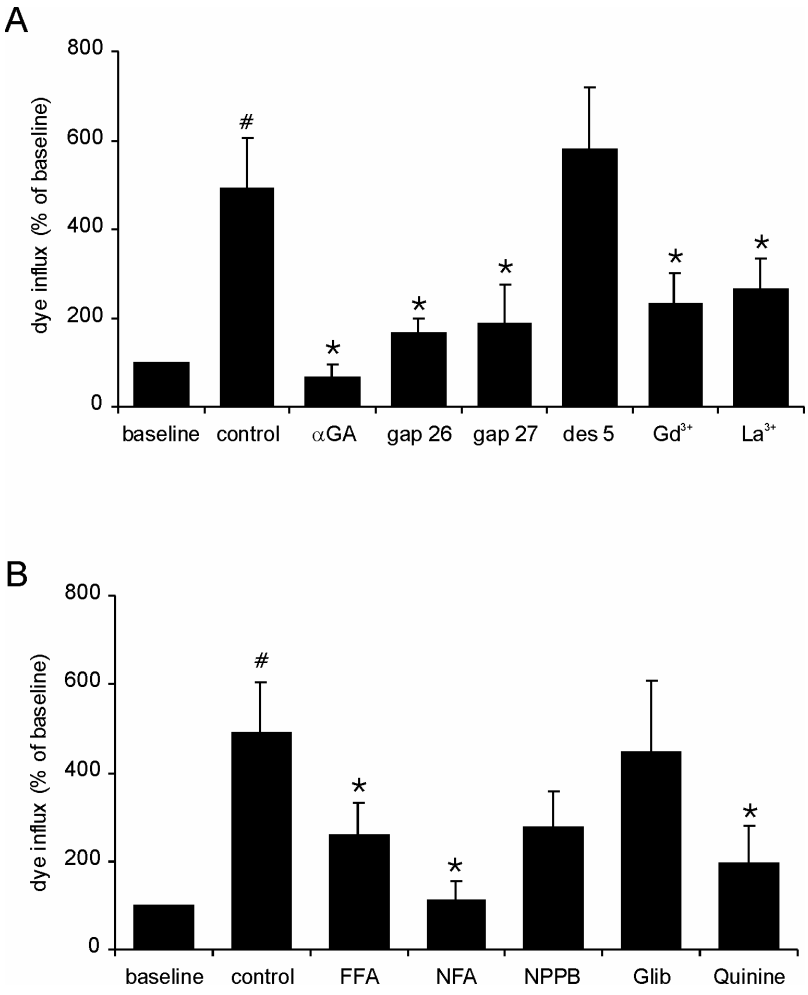

Fig. 6.6 *Propidium iodide uptake triggered by zero extracellular calcium. A. Dye influx was normalized to the dye influx in normal extracellular calcium conditions (baseline). Switching to a zero calcium buffer (control) induced dye influx that was significantly above baseline. Zero calcium-triggered dye uptake was significantly inhibited when the cells were pretreated with α-GA, gap 26, gap 27, Gd^{3+} and La^{3+}, while des 5 had no effect. B. FFA, NFA and quinine significantly reduced zero calcium-triggered dye uptake; NPPB decreased it to a lesser extent and no effect was observed with glibenclamide. #p<0.005, significantly different from baseline; *p<0.05, significantly different from control with n at least 4.*

6.3.4. Cell-to-cell coupling

We performed measurements of the degree of cell-to-cell coupling to assess the effects of the various inhibitory agents used in the previous experiments, using the fluorescence recovery after photobleaching (FRAP) technique. Only α–GA, FFA and NPPB demonstrated a significant inhibition of intercellular coupling, while none of the other substances, including the connexin mimetic peptides gap 26 and gap 27 (applied 30 minutes), had any effect (Fig. 6.7).

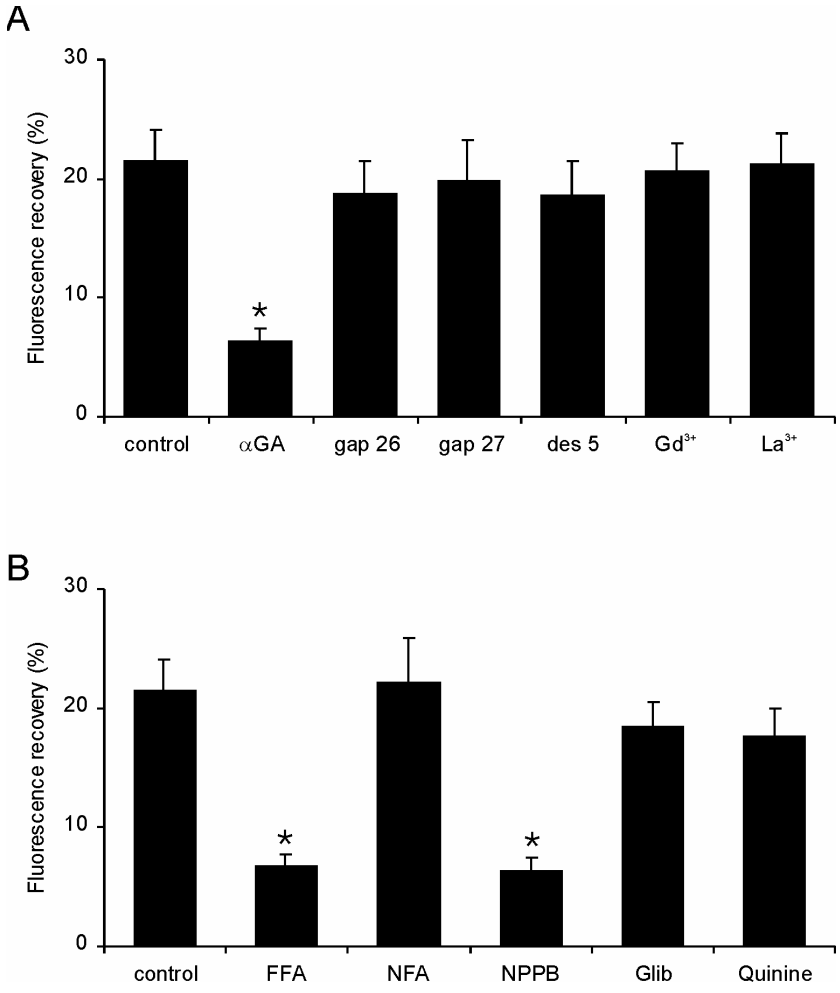

Fig. 6.7 *Gap junctional coupling studied by fluorescence recovery after photobleaching (FRAP). A. α-GA significantly reduced fluorescence recovery, while non of the other agents used had an effect. B. FFA and NPPB showed a significant inhibition of gap junctional coupling. Treatment with NFA, glibenclamide and quinine had no effect. *p<0.05, significantly different from control with n at least 11.*

6.4. Discussion

The purpose of this study was to investigate the mechanisms of ATP release triggered by an increase of intracellular $InsP_3$ or by exposure to zero extracellular calcium, using various pharmacological inhibitors. We found that both $InsP_3$-triggered and zero calcium-triggered ATP release were inhibited by α-GA, the connexin mimetic peptides gap 26 and gap 27, and the trivalent ions, while the zero calcium-triggered ATP release was in addition inhibited by the fenamates and NPPB. Zero calcium-triggered dye uptake was inhibited by the same compounds that inhibited zero calcium-triggered ATP release. Gap 27 only blocked ATP release in connexin-43 transfected cells but not in connexin-32 cells. Gap junctional coupling was blocked by α-GA, FFA and NPPB.

The work was performed on GP8 cells, a brain endothelium cell line (Greenwood *et al.*, 1996), in order to investigate the mechanisms of ATP release triggered by two different stimuli: increasing intracellular $InsP_3$ (Braet *et al.*, 2003b) and exposure of the cells to a zero calcium solution (Stout *et al.*, 2002). A third experimental protocol consisting of zero calcium-triggered dye uptake (Arcuino *et al.*, 2002; Kondo *et al.*, 2000) was included in order to determine whether the ATP release pathway invoked would also allow the transfer of molecules in the inverse direction and to check for consistency with ATP release triggered by the same stimulus. The responses to each of these experimental protocols displayed similarities but also some differences with respect to their sensitivity to the spectrum of pharmacological agents applied in this study.

Connexin hemichannels have been implicated as a possible ATP release pathway (Cotrina *et al.*, 1998a; Stout *et al.*, 2002) and we therefore included α-GA, a gap junction blocker reported to inhibit ATP release in osteoblastic cells (Romanello *et al.*, 2001) in our experiments. In addition, we included two connexin mimetic peptides: gap 26, a synthetic tridecapeptide (13 amino acids) that mimics a sequence on the first extracellular loop of connexin-43 (residue numbers 63-75) containing the SHVR amino acid motif (Rozental *et al.*, 2001b), and gap 27, an undecapeptide (11 amino acids) mimicking a sequence on the second extracellular loop (residue numbers 204-214) of connexin-43 (Evans *et al.*, 2001) that includes the highly conserved SRPTEK sequence (Boitano *et al.*, 2000). Alpha-GA and the connexin mimetic peptides displayed inhibitory effects that were consistent in the $InsP_3$-triggered ATP release experiments and in the zero calcium-triggered ATP release and dye influx experiments. One notable difference is that while α-GA blocked gap junctional coupling, as expected, the peptides did not have any effect on coupling. Connexin mimetic peptides are hypothesized to block gap junctional coupling by preventing connexin-assembly into functional gap junction channels rather than by blocking existing gap junctions (Evans *et al.*, 2001). A possible explanation for the lack of effect of these peptides on gap

junctional coupling observed in our experiments is, first, that the extracellular connexin-loops are connected to the loops of apposed hemichannels and are therefore inaccessible, and second, that disassembly and turn-over of gap junction channels is slow in comparison to the peptide exposure times used (30 minutes).

The lanthanides gadolinium and lanthanum have been demonstrated to inhibit connexin hemichannel currents in both native and connexin-transfected cells (John *et al.*, 1999b; Kondo *et al.*, 2000). The present experiments demonstrate inhibitory effects that were consistently observed over the three experimental protocols used, while displaying no gap junction blocking effects. Trivalent ions are, however, not specific as they block many other channel types, such as calcium channels and mechanosensitive ion channels (Franco *et al.*, 1991; Mlinar *et al.*, 1993), making detailed mechanistic conclusions difficult. The group of fenamates have been demonstrated to block both gap junctional coupling (Harks *et al.*, 2001) and hemichannel currents (Eskandari *et al.*, 2002). In our work, the fenamates FFA and NFA blocked zero calcium-triggered ATP release and dye influx while $InsP_3$-triggered ATP release was not affected. Fenamates have, however, many other effects (blocking cyclo-oxygenase (Boschelli *et al.*, 1993) and several types of ion channels (Gogelein *et al.*, 1990; White *et al.*, 1990) and this might play a role in the differential effects of these compounds observed with the two trigger protocols. Gap junctional coupling was only affected by FFA, which is in contrast with the findings of Harks *et al.* (2001) where NFA was found to be a more potent blocker than FFA. The anion channel blocker NPPB displayed effects that were comparable to the fenamates: no effect on $InsP_3$-triggered ATP release, inhibition of zero calcium-triggered ATP release and dye uptake, and inhibition of gap junctional coupling, an effect not reported before. The potassium channel blockers glibenclamide and quinine have been demonstrated to either block ATP release (glibenclamide; Hashimoto *et al.*, 2001) or potentiate mechanical strain-induced ATP release and dye influx (quinine; Stout *et al.*, 2002). In our hands, neither of these substances had any effect on ATP release, although quinine appeared to inhibit dye influx.

Many of the compounds displaying significant inhibitory effects in the present study, such as α-GA, the trivalents and the fenamates, have been demonstrated to block connexin hemichannels in electrophysiological studies (John *et al.*, 1999b; Quist *et al.*, 2000; Zhang *et al.*, 1998). HeLa cells transfected with either connexin-32 or -43 both displayed an increased baseline and triggered ATP release as compared to wild-type cells, which suggests a role of both connexin-types in ATP release. Gap 27 blocked zero calcium-triggered ATP release in connexin-43 transfected HeLa cells and was without effect on either wild-type or connexin-32 transfected cells. This indicates that the inhibitory effect of gap 27 on triggered ATP release is due to a specific interaction with connexin-43 and not connexin-32.

Taken over all, these data demonstrate consistent inhibitory effects of α-GA, the connexin mimetic peptides and the trivalents on both the $InsP_3$-and zero calcium-triggered ATP release (Fig. 6.8), indicating that the ATP release pathways *activated* with both stimuli share similar properties and involve a shared mechanism. The effects of α-GA, connexin mimetic peptides, trivalents and fenamates on zero calcium-triggered dye influx paralleled the effects on zero calcium-triggered ATP release. This suggests that the zero calcium-triggered dye influx proceeds through a mechanism that is similar to the ATP release mechanism. This mechanism appears to be bidirectionally permeable, has a Q_{10} value that is compatible with free diffusion and thus indicates the involvement of a large conductance pathway or channel. The selective inhibitory effect of gap 27, only observed in connexin-43 transfected HeLa cells, strongly points towards connexins being involved in the release pathway, presumably in the hemichannel configuration which are large conductance channels (Verselis *et al.*, 2000).

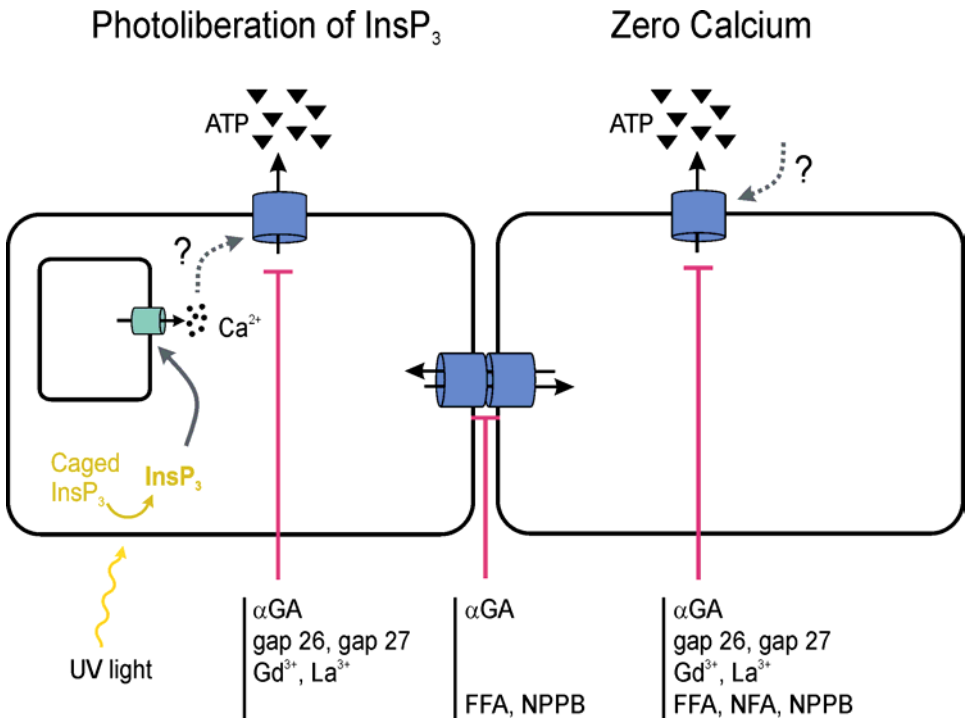

Fig. 6.8 *Summary of the actions of the different agents used in this study on triggered ATP release and gap junctional coupling. ATP release was triggered by photoliberation of $InsP_3$ (from caged $InsP_3$) in the cell interior (cell at the left) or by exposure to a zero extracellular calcium solution (cell at the right). $InsP_3$ triggers calcium release from intracellular stores by activation of $InsP_3$-sensitive channels (green channel). The blue channel connecting the two cells is a gap junction channel, composed of two hemichannels. Photoliberation of $InsP_3$ and exposure to zero extracellular calcium both triggered ATP release but the exact signaling pathway is not known (dashed arrows with question mark). The results with the agents used in this study (indicated on the figure), together with the data obtained in connexin-transfected cells (not shown in this figure) point towards the involvement of connexins in the release process, presumably in the hemichannel conformation (blue channels mediating ATP release).*

The fenamates and NPPB only inhibited zero calcium-triggered ATP release, which points to differences between the two triggers. It is not clear how two such disparate stimuli, one inducing a necessary calcium increase at the inside of the cell (Braet *et al.*, 2003b) and the other decreasing calcium at the outside, might transduce to the same consequence namely the opening of a large conductance channel like the connexin hemichannel. In addition, an increase in intracellular calcium has been well documented to block gap junction channels (Bruzzone *et al.*, 1994; Lazrak *et al.*, 1993; Spray *et al.*, 1992) making the opening of connexin hemichannels difficult to understand. It should, however, be taken into account that the blocking effect of increased calcium requires long exposures to high concentrations of calcium (Lazrak *et al.*, 1993), and is not universal as opposite effects have also been reported (Delage *et al.*, 1998). Further work will be directed to elucidate the details of the signaling cascade leading to ATP release.

In conclusion, our data first show that brain capillary endothelial cells release ATP in response to various triggers. The released ATP might have profound effects on the permeability of the blood-brain barrier (Abbott, 2000), on the vascular tone (Janigro *et al.*, 1997), on astrocytes (Guthrie *et al.*, 1999) and possibly also on blood immune cells (Oviedo-Orta *et al.*, 2002). Second, $InsP_3$- and zero calcium-triggered ATP release is strongly affected by α-GA, connexin mimetic peptides and trivalent ions, suggesting a shared release mechanism for both triggers. Connexin mimetic peptides become increasingly popular to block gap junctional coupling. Our results clearly show that these substances have drastic effects on ATP release, an effect that should be taken into account when interpreting the results obtained in work with these peptides. Finally, the effects of the connexin mimetic peptide gap 27 were demonstrated to be specific for connexin-43, adding evidence to the involvement of connexin hemichannels in ATP release.

chapter

7

Tumor necrosis factor alpha inhibits purinergic calcium signaling in blood-brain barrier endothelial cells

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Abstract

The breaching of the blood-brain barrier is an essential aspect in the pathogenesis of neuroinflammatory diseases, in which TNF-α as well as endothelial calcium ions play a key role. We investigated whether TNF-α could influence the communication of calcium signals between brain endothelial cells (GP8 and RBE4). Intercellular calcium waves triggered by mechanical stimulation or photoliberation of $InsP_3$ in single cells were significantly reduced in size after TNF-α exposure (1000 U/ml, 2 and 24h). Calcium signals are communicated between cells by means of gap junctional and paracrine purinergic signaling. TNF-α significantly inhibited gap junctional coupling and concentration-dependently blocked the triggered component of ATP release. The cytokine displayed similar effects on the uptake of a fluorescent reporter dye into the cells. Previous work with connexin mimetic peptides demonstrated that the triggered ATP release in these cells is connexin-related; these peptides did however not influence ATP release caused by TNF-α. We conclude that TNF-α depresses calcium signal communication in blood-brain barrier endothelial cells, by reducing gap junctional coupling and by inhibiting triggered ATP release. The cytokine thus inhibits connexin-related communication pathways like gap junctions and connexin hemichannels.

7.1. Introduction

The brain is composed of neurons and glial cells and, while neurons are electrically excitable cells, glial cells like astrocytes form a calcium excitable cell population, i.e. they respond to certain stimuli with a calcium response that is communicated to surrounding cells as a calcium wave (Scemes, 2000). Brain endothelial cells are in close contact with astrocytes at the blood-brain barrier, and these cells communicate calcium signals between each other in a homotypic and heterotypic manner (Braet *et al.*, 2000b; Leybaert *et al.*, 1998). Calcium signals between astrocytes and endothelial cells form a bi-directional communication pathway, transferring blood-borne signals to the neuropil, thereby possibly affecting synaptic function (Haydon, 2001), and carrying astrocytic calcium signals to the blood-brain barrier, possibly modulating its transport and barrier functions (Braet *et al.*, 2000b).

The breaching of the blood-brain barrier, i.e. the loss of inter-endothelial tight junctions, is an essential aspect in the pathogenesis of neuroinflammatory diseases like multiple sclerosis and AIDS dementia (Petito *et al.*, 1992; Poser, 1993). There is now ample evidence that cytokines like tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL1-β) and interferon gamma (IFN-γ) released from lymphocytes, macrophages and many other cell types, play a key role in the opening of the barrier (Anthony *et al.*, 1997; de Vries *et al.*, 1996; Munoz-Fernandez *et al.*, 1998). These cytokines stimulate the expression of the cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on brain endothelium, thereby facilitating lymphocyte adherence. The subsequent lymphocyte-endothelial interaction activates diverse signaling cascades: a protein kinase C (PKC) and tyrosine kinase pathway, acting at occludins and the zonula occludens-1 (ZO-1) accessory protein to disrupt tight junctions, and a Rho based pathway activating the actin cytoskeleton and tearing the tight junction proteins away from the cell-cell interface (Bolton *et al.*, 1998; Friedrich *et al.*, 2002; Pfau *et al.*, 1995). An increase in endothelial cytoplasmic calcium concentration is, in addition to this, central to the opening of the blood-brain barrier (Abbott, 2000; Brown *et al.*, 2002; Etienne-Manneville *et al.*, 2000; Wolburg *et al.*, 2002). We hypothesize that calcium signals communicated between endothelial cells may act to spatially spread and thus amplify the calcium-induced opening of the blood-brain barrier. In the present study we asked the question whether the cytokine TNF-α influences the communication of calcium signals between endothelial cells, thereby affecting the postulated spatial spread of blood-brain barrier opening.

Our work shows that the cytokine depresses endothelial calcium signal communication by inhibiting gap junctional coupling, by increasing cellular ATP release while blocking the triggered component of ATP release. TNF-α thus seems to limit the spread of calcium signals at the blood-

brain barrier by various mechanisms. Because the cytokine affects the first and most essential step of the paracrine ATP signaling pathway, i.e. the phase of ATP release, the TNF-α actions are expected not to be limited to endothelial cell communication but may also influence paracrine communication to other cell types in the neighborhood such as blood cells, astrocytes and smooth muscle cells. Finally, TNF-α appears to affect two connexin-related pathways in a parallel manner: it reduces gap junctional communication (connexin-channels) and blocks triggered ATP release, which in previous work was demonstrated to be related to the opening of connexin hemichannels (Braet *et al.*, 2003b; Braet *et al.*, 2003c) (connexin hemichannels are reviewed by Goodenough *et al.* (2003).

7.2. Materials

Fluo-3 acetoxymethyl ester (fluo-3 AM), D-myo-inositol 1,4,5-trisphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester trisodium salt (NPE-caged $InsP_3$), DMNB-caged fluorescein dextran (MW 3000), propidium iodide (MW 668), 6-carboxyfluorescein (MW 376), dextran tetramethylrhodamine (MW 10000) and dextran fluorescein (MW 70000) were obtained from Molecular Probes (Leiden, The Netherlands). Tumor necrosis factor alpha (TNF-α), the ATP assay kit, phorbol 12-myristate 13-acetate (PMA) and adenosine 5' trisphosphate disodium salt (ATP) were from Sigma (Bornem, Belgium). The peptide GAP 26 (VCYDKSFPISHVR) was a kind gift of Prof. W.H. Evans (Dept. of Medical Biochemistry, University of Wales College of Medicine, Cardiff, UK). It was synthesized by solid phase chemistry and purified by HPLC and purity (95 %) assessed by HPLC.

7.3. Methods

7.3.1. Cell cultures

In this study we used two rat brain endothelial cell lines: RBE4 (a kind gift of Dr. F. Roux, Neurotech SA, Evry, France) (Roux *et al.*, 1994) and GP8 (GP8/3.9; Greenwood *et al.*, 1996). Culture media (all from Gibco, Merelbeke, Belgium) were alpha-MEM/Ham's F10 (1:1) with 10 % fetal bovine serum (FBS), 1 µg/ml bFGF (Boehringer Mannheim, Brussels, Belgium) and 0.3 mg/ml geneticin (Gibco, Merelbeke, Belgium) for RBE4 and GP8. Cells were grown on glass-bottom Petri dishes (MatTek Corporation, Ashwood, MA, USA) coated with collagen type I (Boehringer Mannheim, Brussels, Belgium) and used for experiments upon confluency. TNF-α was added to the culture medium and applied to the cells incubated under culture conditions (humidified 5 % CO_2) for the time periods used (2h or 24h). Matched control cultures were treated with culture medium not containing TNF-α in the same manner as the ones that received the cytokine.

7.3.2. Calcium imaging

Cytoplasmic free calcium was measured using the calcium-sensitive dye fluo-3 in combination with epifluorescence video microscopy and digital imaging. Cell cultures were loaded with fluo-3 for 1h at room temperature in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4) containing 10 µM fluo-3 AM (Molecular Probes, Leiden, The Netherlands) and 1 mM probenecid. Cultures were then washed with HBSS-HEPES and left at room temperature for 30 min for de-esterification. HBSS-HEPES was the bathing solution for all calcium imaging

experiments. Cells were viewed with an inverted epifluorescence microscope (Nikon Eclipse TE300, Analis, Ghent, Belgium) using a x 40 oil immersion lens (CFI Plan Fluor, Nikon). Fluo-3 fluorescence images were obtained by excitation at 485 nm, reflection off a dichroic mirror with cut-off at 510 nm, and emission bandpass filtering at 535 nm (485DF22, 505DRLPXR and 535DF35 filters respectively from Omega Optical, Brattleboro, VT). Images were captured using an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) and stored on an S-VHS video recorder (Panasonic, Avicom, De Pinte, Belgium) or directly to a PC equipped with an image acquisition and processing board (DT3155, Data Translation, Marlboro, MA). Each experiment was concluded by recording background images in a Petri dish containing only medium and no cells; background images were subtracted from the fluo-3 fluorescence image sequences.

7.3.3. Loading of caged $InsP_3$ by electroporation and UV spot illumination

The cells were loaded with caged $InsP_3$ by electroporation. Cultures were briefly rinsed with electroporation buffer (300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1 mM $MgCl_2$ and 2 mM HEPES, pH 7.20) and thereafter a small volume (5 µl) of caged $InsP_3$ (200 µM) in electroporation buffer was added. This solution contained in addition dextran rhodamine (100 µM) to visualize the electroporation zone. Electroporation was done on the stage of an inverted microscope with a parallel wire electrode positioned close to the cell surface, as described in detail in Braet *et al.* (2003b). The zone of caged $InsP_3$ loaded cells was estimated to be approximately 1.3 mm wide and 10 mm long. UV *spot* illumination for photorelease in calcium imaging experiments was performed with a Hg-arc lamp coupled to the microscope epifluorescence input, as described before (Braet *et al.*, 2003b; Leybaert *et al.*, 2001). The UV spot had a half-energy diameter of 10 µm, as determined by flashing a thin layer of DMNB-caged fluorescein dextran (MW 3000) at 3 mM mixed (1:1) with Dako Glycergel (Dako Corporation, Carpinteria, CA).

7.3.4. Mechanical cell stimulation

Mechanical stimulation of a single cell was performed by gentle stimulation with a glass microneedle (2 µm tip size) mounted on a piezo-electric device and driven by a single pulse of 100 ms duration to produce a tip displacement of approximately 5 µm.

7.3.5. Apoptotic index

The apoptotic index was determined in cell cultures that were fixed for 20 min in 4 % formaldehyde and stained with DAPI (1/10000) for 5 min. The cells were washed 3 times with PBS after each step. The apoptotic nuclei were counted in 6 frames each containing approximately 170 cells.

7.3.6. Scrape loading and dye-transfer

Gap junctional coupling was investigated with the scrape loading and dye-transfer technique (Blomstrand *et al.*, 1999; Opsahl *et al.*, 2000). Confluent cultures of GP8 and RBE4 were placed in a nominally calcium-free saline consisting of 137 mM NaCl, 5.36 mM KCl, 0.81 mM $MgCl_2$, 5.55 mM D-glucose and 25 mM HEPES (pH 7.4) containing 0.4 mM 6-carboxyfluorescein for 10 min before applying a linear scratch across the culture with a syringe needle and left for another 10 min in the same solution. The cultures were then washed 3 x with HBSS-HEPES and left for 30 min to recover. The cells were viewed as described above and images were processed with custom-made software to acquire a fluorescence diffusion profile. This profile was fitted to a mono-exponential function using a non-linear least square fitting in order to obtain a spatial constant of lateral intercellular diffusion.

7.3.7. Extracellular ATP measurements

ATP release in response to photoliberation of $InsP_3$ was determined in GP8 and RBE4 cultures using an ATP bioluminescent luciferin/luciferase assay kit (product no. FL-AA, Sigma, Bornem, Belgium). The photolytic UV light in these experiments was applied as a *field* illumination (not spot illumination) exposing multiple cells. To that purpose, light from a Hg-arc lamp with quartz collector lens was directed through a shutter, a 330 nm bandpass filter and a mirror (10D510AL.2, Newport, Leuven, Belgium) to form an image of the arc at the level of the cell culture. The total energy dose per unit of surface area applied in UV field illumination experiments was adjusted so that it was equal to the energy dose per unit of surface in the spot illumination experiments, as described in detail in Braet *et al.*, 2003b. The UV exposure time for field illumination was 2 seconds determined in this way. The photolytic efficiency with this exposure time was in the order of the quantum efficiency of caged $InsP_3$ (0.65), indicating that the entire amount of caged probe present in the cultures was effectively photocleaved upon a single field illumination exposure. All experimental groups received the photolytic light. Immediately after UV exposure, 100 µl of the supernatant was collected from the cell cultures and transferred to 100 µl ATP assay mix solution (used at 5-fold dilution). Light emission from the 200 µl mix was then measured with a custom build luminometer consisting of a photomultiplier tube (9924B, Thorn-Emi Electron Tubes, Middlesex, UK). Luminescence was determined from the photomultiplier current that was registered with custom made computer software. ATP release was also measured in response to short (2 min) exposure of the cells to zero extracellular calcium conditions (calcium- and magnesium-free HBSS-HEPES with 1 mM EGTA added). The cells received 200 µl of this solution and after 2 min 100 µl of the supernatant was collected and transferred to 100 µl ATP assay mix solution (used at 5-fold dilution).

7.3.8. Propidium iodide uptake

The zero extracellular calcium condition used for ATP release was also employed to trigger propidium iodide uptake into the cells (Arcuino *et al.*, 2002; Braet *et al.*, 2003c). Cell cultures received the zero calcium solution containing 2 mM propidium iodide for 10 min at room temperature, were washed 3 x with HBSS-HEPES, and pictures were then acquired with a x10 objective, TRITC epifluorescence settings and a cooled CCD camera (SensiCam, PCO, Kelheim, Germany). In each culture, 10 images were taken in which average dye uptake was determined. Propidium iodide positivity was quantified by calculating the number of pixels with a fluorescence intensity above a threshold value. Comparison between manually counted propidium iodide positive cells and supra-threshold pixels showed a linear relation between both parameters. The number of cells demonstrating zero calcium-triggered propidium iodide uptake amounted to 13.0 $\pm$ 0.9 % of the cells (n=10). Separate control experiments showed that zero calcium exposure caused, in addition to propidium iodide uptake, also uptake of small dyes like 6-carboxyfluorescein (MW 376) while excluding larger dyes like dextran tetramethylrhodamine 10 kDa or dextran fluorescein 70 kDa.

7.3.9. Data analysis and statistics

Calcium changes were determined as relative fluo-3 fluorescence changes, i.e. $\Delta F/F_0 = [F - F_0] / F_0$ with F_0 the fluorescence before stimulation and F the time-dependent fluorescence signal after stimulation. The extent of cell-to-cell propagation of calcium changes was determined from the size of the intercellular calcium wave at its maximal state of extension and was quantified by determining the surface area where $\Delta F/F_0$ was above a threshold of 50 %. We determined the surface area rather than the wave radius because it is a much more sensitive parameter to detect changes in the extent of calcium signal propagation (changes are amplified quadratically as compared to the radius). Average $\Delta F/F_0$ changes for the whole calcium waves were determined as the average calculated from all pixels that showed an above threshold change. Curve fittings for dose-response relations were performed with non-linear least square procedures available in the program Inplot. The data are expressed as mean ± s.e.m. with n denoting the number of experiments. Statistical significance was tested using a t-test for unpaired observations and using a p value of less than 0.05. Multiple groups were compared using variance analysis followed by the Dunnett test for multiple comparisons to the control group or the Student-Newman-Keuls test for comparison of all groups among each other.

7.4. Results

7.4.1. TNF-α reduces the size of intercellular calcium waves

Cell-to-cell calcium signal communication was investigated by triggering intercellular calcium waves in confluent cultures of RBE4 and GP8 brain capillary endothelial cell lines. Intercellular calcium waves were triggered either by mechanical cell stimulation or by photoliberating $InsP_3$ in a single cell. Gentle mechanical stimulation of a single cell triggered an intercellular propagating calcium wave in the two cell lines. The calcium wave size, determined as the surface area of cells showing an above threshold calcium change ($\Delta F/F_0$ > 50 %) at maximal wave extension, averaged 17448 ± 1162 μm^2 (n=23) in RBE4 cells and 30774 ± 1254 μm^2 (n=55) in GP8 (corresponding to a wave radius of approx. 75 and 99 µm respectively). Treating the cultures with TNF-α for 2h (1000 U/ml) significantly reduced the size of intercellular calcium waves to approximately 80-90 % of their control size (Fig. 7.1 & Fig. 7.2A). Extending the TNF-α exposure to 24h further reduced the wave size to approx. 60-75 % of control.

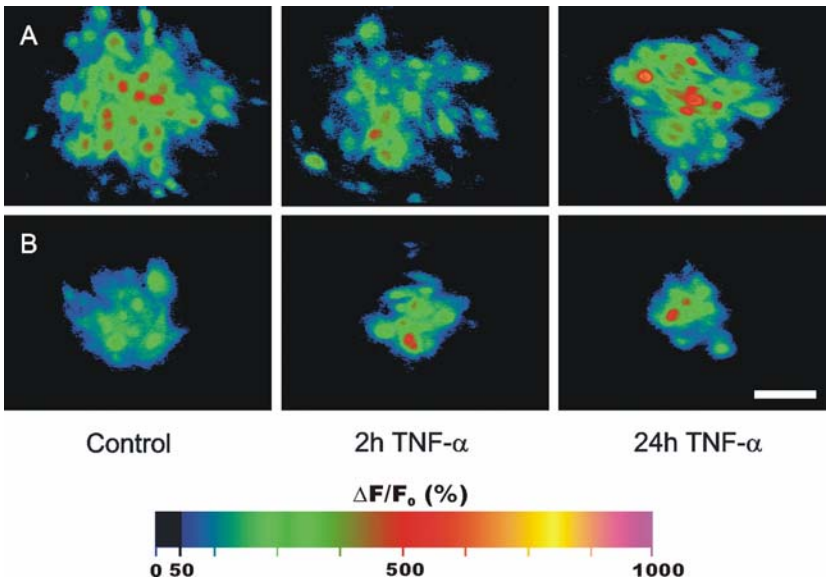

Fig. 7.1 *Effect of TNF-α on intercellular calcium waves in representative experiments performed on confluent GP8 cultures. A. Calcium waves triggered by mechanical stimulation of a single cell. B. Calcium waves triggered by photoliberation of $InsP_3$ in a single cell. TNF-α (1000 U/ml) reduced the extent of intercellular calcium wave propagation for both stimulation types and for both short (2h) and longer (24h) exposure times. The images depict relative fluo-3 fluorescence changes ($\Delta F/F_0$) color coded as indicated in the color bar; sub-threshold pixels ($\Delta F/F_0$ < 50 %) are given in black. The calcium waves are each shown at their maximal state of extension. Note that the $InsP_3$-triggered calcium waves are smaller than the mechanically triggered waves. All calcium waves shown were recorded in separate cell cultures. The white calibration strip measures 100 µm.*

Photoliberating $InsP_3$ in a single cell triggered intercellular calcium waves that were significantly smaller than waves triggered by mechanical stimulation (5688 ± 159 μm^2 [n=35] for RBE4 and 8944 ± 302 μm^2 [n=57] for GP8, $p<0.0001$; wave radii corresponding to these areas are respectively 42 and 53 µm). Two hours exposure to TNF-α significantly reduced the calcium wave size to an extent comparable with the observations in the mechanical stimulation experiments, and 24h TNF-α slightly reduced it further (Fig. 7.2B). Pooling the data for the two cell lines and for the two stimulation protocols showed that the calcium wave following 24h TNF-α was significantly smaller as compared to the 2h condition (relative wave size of 100.0 $\pm$ 1.8 % under control conditions, 80.8 $\pm$ 3.0 % with 2h TNF-α and 70.0 $\pm$ 2.1 % with 24h TNF-α; all values significantly different from each other with p at least smaller than 0.01; n=124). TNF-α exposure had no significant effect on the amplitude of the calcium changes associated with the calcium wave ($\Delta F/F_0$ attained 93.4 $\pm$ 3.1 % of a control amplitude of 100.0 $\pm$ 3.0 % following 2h TNF-α and 97.1 $\pm$ 3.6 % for 24h treatment; pooled data for the two cell lines and for the two stimulation protocols; n=124). TNF-α is known to trigger apoptosis in many cell types when applied at a high dose (Peiretti *et al.*, 1997), and to determine whether the observed reduction in wave calcium wave size was perhaps due to the induction of apoptosis, we counted apoptotic nuclei in RBE4 cells. TNF-α did not affect the apoptotic index in RBE4 cells (0.2 ± 0.1 % in control condition and 0.3 ± 0.1 % after 24h TNF-α, n=4).

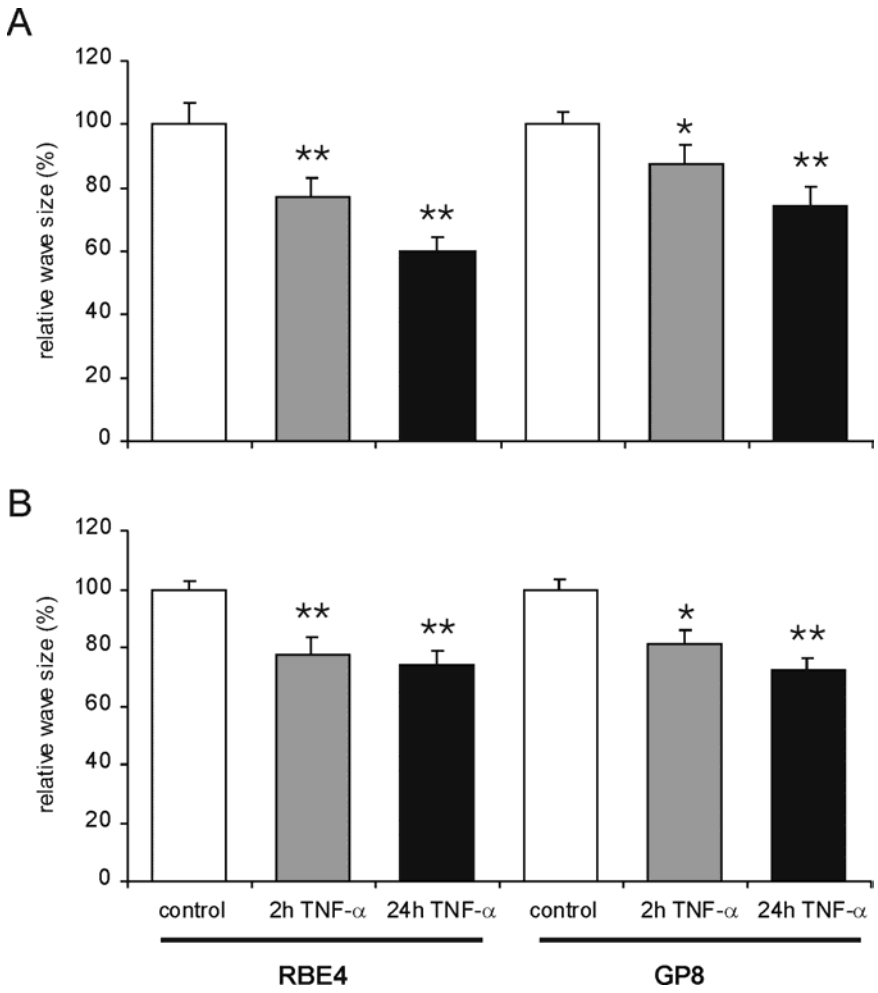

Fig. 7.2 *Averaged data summarizing the effect of TNF-α on the extent of intercellular calcium wave propagation. A. Effect on mechanically triggered calcium waves. B. Effect on $InsP_3$-triggered calcium waves. Two hours TNF-α treatment (1000 U/ml) significantly reduced the extent of intercellular calcium wave propagation (calcium wave size) in both RBE4 and GP8 cultures and this effect was more pronounced after 24h treatment. The calcium wave size was determined from the surface area of cells showing an above threshold calcium change ($\Delta F/F_0$ > 50 %) at maximal wave extension and is expressed as the wave size relative to the control wave size. *p<0.05, **p<0.01 as compared to control (Con); n=23-57.*

7.4.2. TNF-α reduces gap junctional coupling

Intercellular calcium waves in endothelial cells can be communicated by an intracellular/gap junction dependent pathway and an extracellular/purinergic pathway, and TNF-α can thus be hypothesized to influence both pathways (Braet *et al.*, 2001; Demer *et al.*, 1993; Moerenhout *et al.*, 2001). We investigated the effect of TNF-α on gap junctional coupling with the scrape loading and dye transfer method. Control GP8 cultures had a spatial constant of intercellular 6-carboxyfluorescein spread of 48.5 ± 9.0 μm (n=8) and this parameter was significantly smaller in

RBE4 (10.0 ± 1.0 µm [n=10]; p<0.01). In both cell types 2h TNF-α treatment significantly reduced the spatial constant of intercellular dye spread to approximately half its control value (Fig. 7.3 A & B). Twenty-four hour TNF-α treatment further reduced the spatial constant and this was most clear in GP8. TNF-α thus significantly reduced the gap junctional coupling of the cells and the effect paralleled the effects of the substance on intercellular calcium waves.

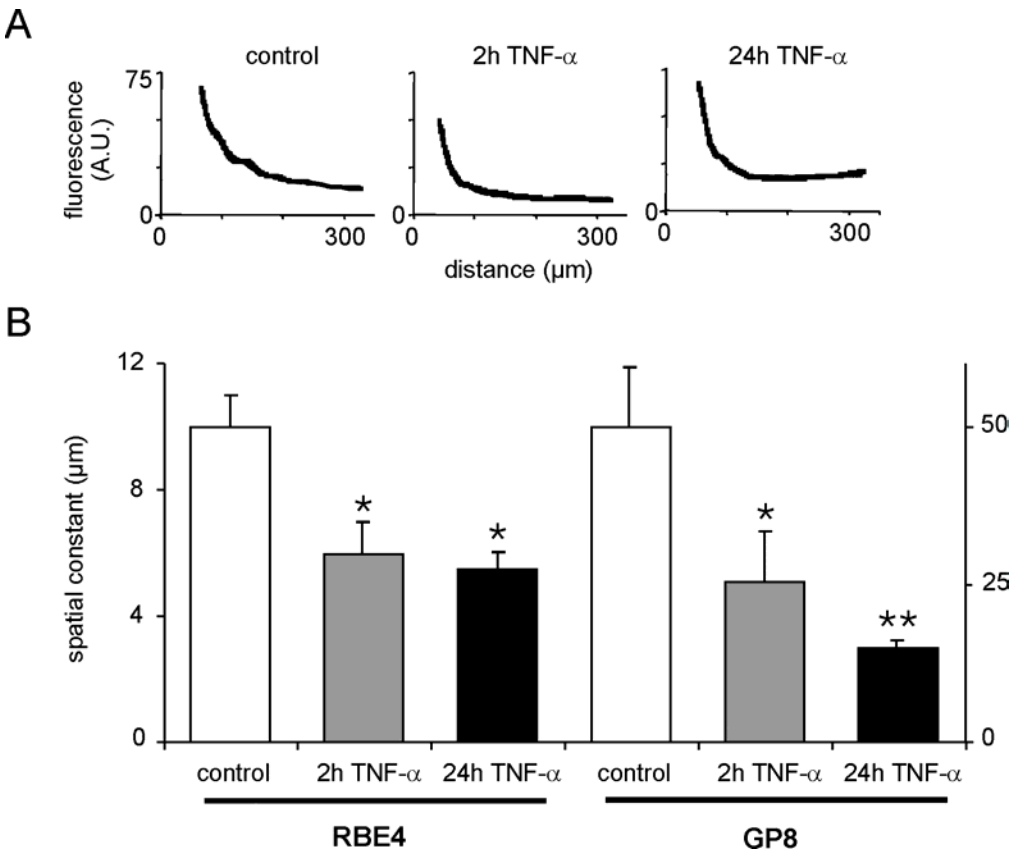

Fig. 7.3 *Effect of TNF-α on gap junctional coupling. A. Example of dye diffusion profiles away from the border of the scrape loading zone, under control and TNF-α (1000 U/ml) treated conditions in GP8 cells. The cell-to-cell spread of the 6-carboxyfluorescein dye (fluorescence expressed in arbitrary units [A.U.]) was quantified by determining the spatial constant of a mono-exponential diffusion profile (fine line indicates fitted curve). B. Averaged data demonstrate that TNF-α significantly reduced the spatial constant of intercellular diffusion to approximately half its control value in both RBE4 and GP8 cells when applied for 2h. In GP8 cells 24h treatment further reduced the coupling. *p<0.05, **p<0.01 as compared to control (Con); n=8-10.*

7.4.3. TNF-α inhibits $InsP_3$-triggered ATP release

The inhibitory effect of TNF-α on intercellular calcium waves can, in part, be explained by the effect of the cytokine on gap junctional coupling, but it is also possible that it influences the extracellular/purinergic pathway of calcium signal communication. We investigated the effect of TNF-α on the two step process of paracrine signaling: the first step involving the release of the purinergic messenger ATP by the cells and the second step consisting of the response of the cells to the extracellulary diffusing ATP. To test the effect of TNF-α on the last step, we performed ATP superfusion experiments and probed the effect of the cytokine on the calcium transients induced in this way. Three minutes superfusion with HBSS-HEPES were allowed to record background calcium signal. TNF-α did not influence the amplitude of the calcium transients triggered by short exposure (1 min) to 10 µM ATP ($\Delta F/F_0$ reached 95.6 ± 7.2 % of a control amplitude of 100.0 ± 5.5 % following 2h TNF-α and 103.0 ± 6.9 % for 24h treatment; pooled data for the two cell lines; n=13). TNF-α was also tested for its effects on the first step of paracrine signaling, i.e. on the release of ATP by the cells. Cellular ATP release was triggered by photoliberating $InsP_3$ and recorded by using a bioluminescent assay. We used direct photolytic stimulation with $InsP_3$ instead of applying an agonist acting through the $InsP_3$ pathway in order to exclude known effects of TNF-α on agonist-triggered phosphoinositide production (Yorek *et al.*, 1999). Cells loaded by electroporation with vehicle only produced baseline ATP release. When caged $InsP_3$ was added to the loading medium, the ATP response to photoactivation was significantly higher as compared to baseline, demonstrating the presence of a net $InsP_3$-triggered component of ATP release (Fig. 7.4). In cultures exposed for 2h to TNF-α, baseline ATP release was significantly elevated as compared to control conditions. The ATP response to photorelease of $InsP_3$ was not different from baseline ATP release under these conditions, indicating that the net component of $InsP_3$-triggered ATP release is suppressed by TNF-α (Fig. 7.4). Twenty-four hour treatment of the cultures showed a similar result: increased baseline release and an $InsP_3$-triggered ATP response not different from baseline, indicating again suppression of the $InsP_3$-triggered ATP release component.

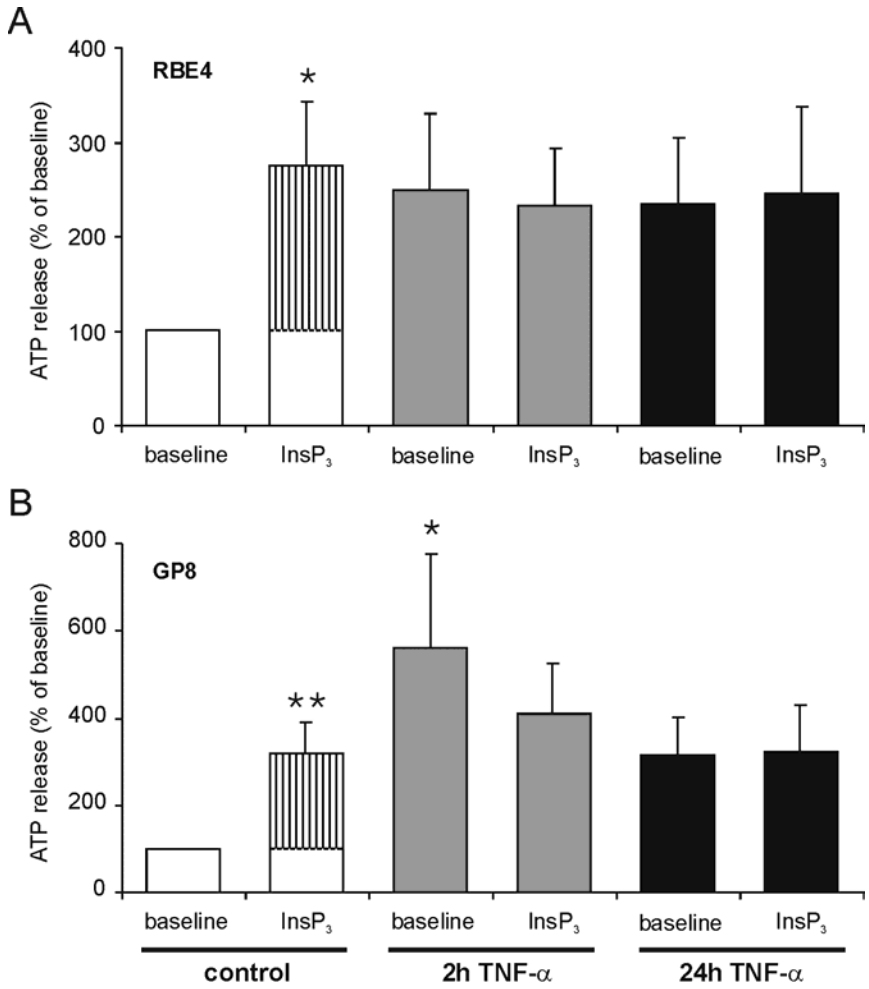

Fig. 7.4 *Effect of TNF-α on ATP release triggered by photoliberation of $InsP_3$. The first open bar shows baseline ATP release in cells loaded with vehicle only and kept under control conditions. The second open bar ($InsP_3$) depicts photoactivation-triggered ATP release from cells loaded with caged $InsP_3$ and kept under control conditions. The net component of $InsP_3$-triggered ATP release can be estimated from the difference between the two bars (vertically hatched portion). The grey bars depict ATP release following 2h TNF-α (1000 U/ml) and demonstrate increased baseline ATP release (as compared to baseline under control conditions) and a reduced $InsP_3$-triggered component as can be inferred from the equal height of the baseline and $InsP_3$ bars. The black bars depict ATP release following 24h TNF-α, again demonstrating stimulated baseline release and disappearance of the net component of $InsP_3$-triggered release. These results were very comparable in the two cell lines used (RBE4 and GP8). All bars are expressed relative to the first bar (baseline under control conditions). * = $p<0.05$, ** = $p<0.01$ as compared to baseline; the vehicle and $InsP_3$ bars under TNF-α conditions are not significantly different from each other; n=9 for RBE4 and 13 for GP8.*

7.4.4. TNF-α inhibits zero calcium-triggered ATP release and dye uptake

ATP release by the cells has been suggested to be consistent with the opening of connexin hemichannels (Braet *et al.*, 2003b; Stout *et al.*, 2002), which are channels composed of six connexin-subunits that do not connect to apposed hemichannels (Goodenough *et al.*, 2003). Exposure of the cells to zero extracellular calcium solutions has been used to trigger ATP release through this pathway (Arcuino *et al.*, 2002). We have applied the zero extracellular calcium trigger to investigate the effect of TNF-α on ATP release brought about in this way. Two minutes exposure to zero extracellular calcium conditions (1 mM EGTA) triggered significant ATP release (Fig. 7.5A); TNF-α treatment increased the baseline ATP release and limited the triggered release such that the net component of zero calcium-triggered ATP release disappeared. These effects of TNF-α, both at 2 and 24h exposure, thus parallel the observations with the $InsP_3$ trigger.

Zero calcium conditions have been utilized to demonstrate the opening of plasma membrane channels (presumably connexin hemichannels) and the entry of fluorescent dyes into the cells (Braet *et al.*, 2003c; DeVries *et al.*, 1992; Li *et al.*, 1996). We have employed a similar protocol to test whether TNF-α affects dye uptake. Ten minutes exposure to zero extracellular calcium triggered significant uptake of propidium iodide (MW 562) into the cells while excluding larger dextran-coupled dyes. TNF-α increased the baseline dye uptake and reduced the triggered uptake to the baseline level, indicating suppression of the net component of zero calcium-triggered dye uptake (Fig. 7.5B). These effects of TNF-α are thus similar to the observations made with $InsP_3$- or zero calcium-triggered ATP release.

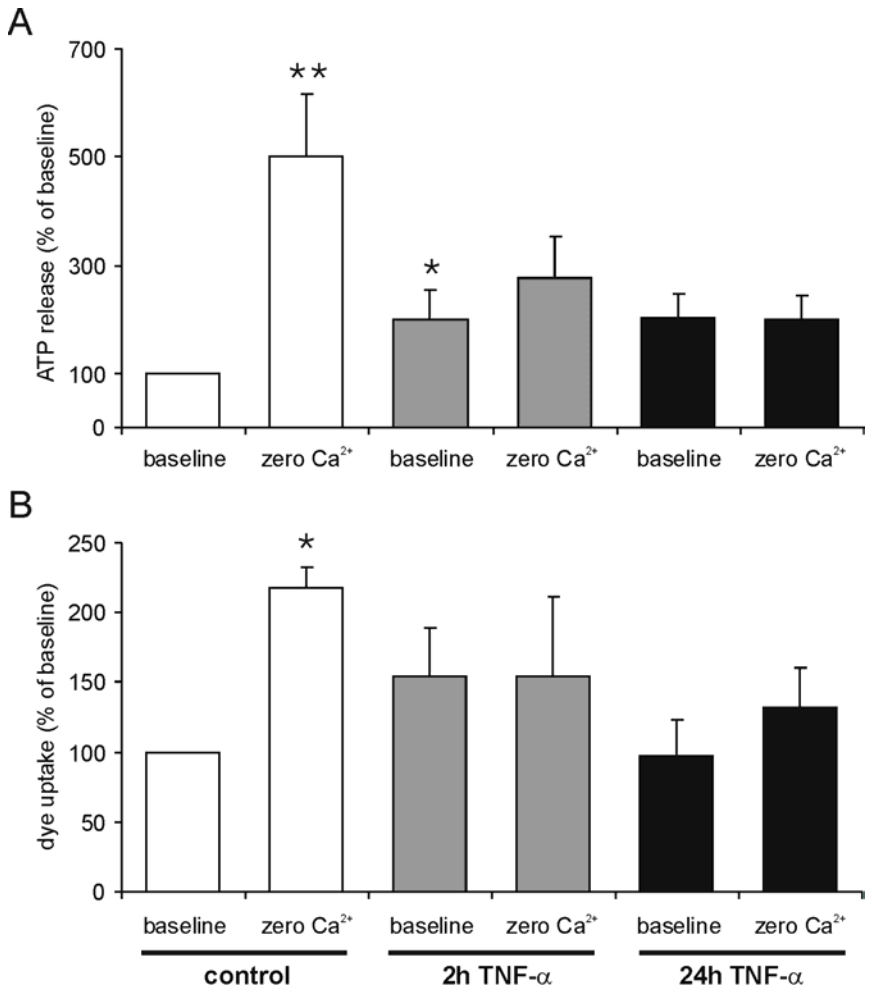

Fig. 7.5 *Effect of TNF-α on ATP release and dye uptake triggered by zero extracellular calcium in GP8 cells. A. Zero calcium-triggered ATP release. Open bars show baseline and zero calcium-triggered ATP release (zero Ca^{2+}). Grey bars depict the effect of 2h TNF-α (1000 U/ml) and black bars the effect of 24h exposure. TNF-α increased the baseline ATP release and inhibited the triggered component of ATP release (difference between baseline and zero Ca^{2+} bars, both in the 2h and 24h exposures). B. Zero calcium-triggered dye uptake (propidium iodide). The bars represent the same conditions as described under A. TNF-α increased the baseline dye uptake and inhibited the triggered component of dye uptake (baseline and zero Ca^{2+} difference). ∗p<0.05, ∗∗p<0.01 as compared to baseline; n=10-20 in A and 6 in B.*

We have previously demonstrated that the triggered component of cellular ATP release and dye uptake in endothelial cells are connexin-dependent phenomena, based on the drastic blocking effect of connexin mimetic peptides like gap 26 or gap 27 (Braet *et al.*, 2003b; Braet *et al.*, 2003c). We tested gap 26 (0.25 mg/ml, 15 min) in the present work to determine whether the TNF-α induced increase in baseline ATP release is also connexin-related. The elevated basal ATP release following TNF-α exposure was not affected by gap 26, suggesting the involvement of a connexin-independent release mechanism (Fig. 7.6A).

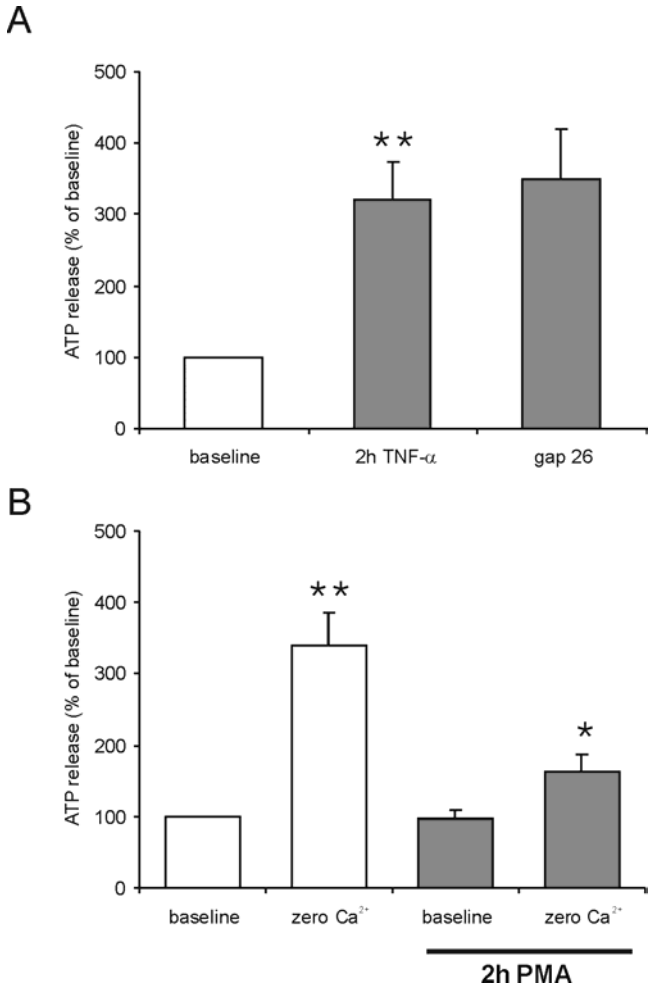

Fig. 7.6 *A. The connexin mimetic peptide gap 26 does not abbrogate TNF-α induced elevation of basal ATP release. Two hours treatment with TNF-α (1000 U/ml) significantly increased the baseline ATP which was not affected by gap 26 (0.25 mg/ml, 15 min). B. PKC stimulation mimics the TNF-α effects on triggered ATP release. PKC stimulation with PMA (10 nM, 2h) inhibited the triggered component of ATP release but did not influence baseline ATP release. *p<0.05, **p<0.01 as compared to baseline; n=4-10 in A and 6–10 in B.*

TNF-α is known to act through activation of kinases like PKC (Brosnan *et al.*, 2001; Ferro *et al.*, 2000; van Rijen *et al.*, 1998). We tested the effect of PKC stimulation with PMA on basal and zero calcium-triggered ATP release. PMA (10 nM, 2h) did not affect basal ATP release but significantly reduced the triggered part of ATP release (Fig. 7.6B). PKC is known to phosphorylate connexins thereby inhibiting gap junctions (Lampe *et al.*, 2000) and also connexin hemichannels (Ngezahayo *et al.*, 1998) involved in triggered ATP release. The activation of baseline ATP release by TNF-α is not connexin-related and the absence of any effect of PKC stimulation on baseline ATP release is thus in line with the expectations.

To determine whether the inhibitory effect of TNF-α on ATP release occurs in a graded manner, we performed concentration-response experiments in which the cells were exposed to various

TNF-α concentrations and where the inhibitory effect on stimulated ATP release was assessed. Both $InsP_3$- and zero calcium-triggered ATP release were tested, and both triggers demonstrated a concentration-dependent inhibitory effect of TNF-α with a half-maximal effect concentration in the order of 1 U/ml (Fig. 7.7). This concentration corresponds to picomolar concentrations, a value that is in good agreement with the half-maximal effect concentrations that have been reported for interaction of the cytokine with the type-1 receptor (1.5-20 pM range) (Coffman *et al.*, 1988; Grell *et al.*, 1998).

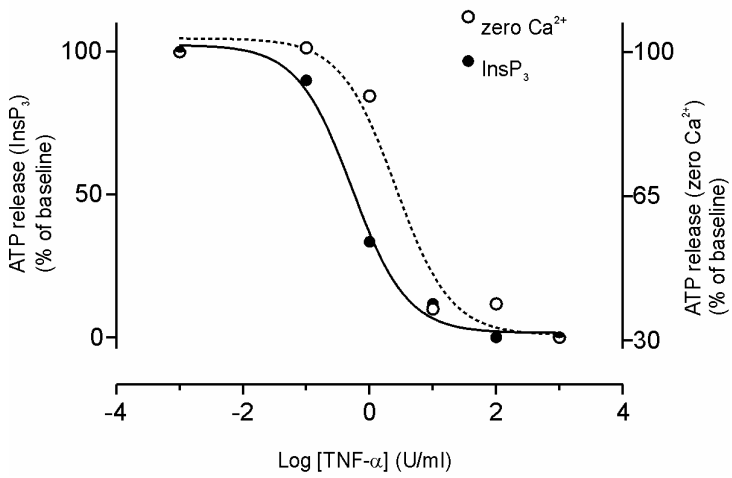

Fig. 7.7 *Concentration-response curve for the inhibitory effect of TNF-α on ATP release in GP8 cells (24h TNF-α exposures). $InsP_3$-triggered and zero calcium-triggered ATP release showed comparable sensitivity to the inhibitory effect of TNF-α, with half-maximal effects in the order of 0.5-2.6 U/mL (n=2 for each condition).*

7.5. Discussion

This study was undertaken to investigate the effect of the proinflammatory cytokine TNF-α on the pathways of calcium signal communication operating between brain endothelial cells. Our results show that TNF-α reduces the extent of intercellular calcium wave propagation, reduces the degree of gap junctional coupling, stimulates basal ATP release, inhibits the release of ATP triggered by $InsP_3$ or zero extracellular calcium, and impedes zero calcium-triggered dye uptake.

Cell-to-cell propagating calcium signals, appearing in monolayer cell cultures as so called intercellular calcium waves, can in general be communicated through two different pathways: an intracellular pathway involving the diffusion of a messenger like $InsP_3$ through gap junctions, and an extracellular pathway implicating the release of a purinergic or glutamatergic messenger, acting in a paracrine way (Blomstrand *et al.*, 1999; Osipchuk *et al.*, 1992). Mechanical cell stimulation and photoliberation of $InsP_3$ in a single cell invokes these two pathways in brain endothelial cells (Braet *et al.*, 2001). TNF-α reduced the size of intercellular calcium waves, both in RBE4 and GP8 brain endothelial cells, to a degree that was comparable for the two stimulation modes and that was more prominent following 24h as compared to 2h exposure. This reduced calcium wave size can be the result from diverse causes that will be further examined.

First, we considered that the cytokine reduces the magnitude of the calcium changes associated with the calcium wave. This possibility could result in a smaller wave size because of a smaller number of suprathreshold pixels. TNF-α has been shown to influence agonist-induced calcium transients, either stimulating transients triggered by potassium chloride, carbachol or AMPA (Amrani *et al.*, 1996; De *et al.*, 2000), inhibiting transients triggered by endothelin (Yorek *et al.*, 1999), or buffering transients due to increased expression of the calcium-binding protein calbindin (Cheng *et al.*, 1994). In our experiments, TNF-α did not affect the amplitude of the calcium changes associated with the intercellular calcium wave, making this possibility unlikely. A second possibility is that TNF-α triggers apoptosis thereby reducing the number of cells contributing to the calcium wave or the cell volume (Okada *et al.*, 2001). The apoptotic index was low in our cultures and TNF-α did not affect it, in line with observations in other types of endothelial cells (van Rijen *et al.*, 1998), making this option invalid. A third possibility is that the cytokine reduces the level of gap junctional coupling, as has been described in other cell types (Chanson *et al.*, 2001; Fernandez-Cobo *et al.*, 1999; van Rijen *et al.*, 1998) and also with other cytokines like IL1-β (John *et al.*, 1999a). This would hamper the intercellular spread of $InsP_3$ and consequently reduce the size of the calcium wave. Our experiments indeed confirm depressed dye coupling after 2h TNF-α treatment that is further reduced following 24h exposure. A last possibility, clearly endorsed by the present results, is that TNF-α affects the purinergic calcium signaling pathway. Other cytokines like

IL1-β have been demonstrated to induce a shift in the P_2Y receptor subtype expression thereby promoting the propagation of calcium signals (John *et al.*, 1999a). Our experiments with TNF-α did not reveal any effects of the cytokine on ATP-triggered calcium transients. This does however not exclude subtle changes in receptor subtype expression, for which more elaborate experiments with specific agonists and antagonists are needed. TNF-α did, however, drastically alter the pattern of cellular ATP release as evidenced by the ATP assay experiments, shifting the focus to the first step of the paracrine signaling cascade.

TNF-α had a differential effect on ATP release: it strongly stimulated baseline release and limited the stimulated release to the (increased) baseline level, i.e. it thus removed the net component of $InsP_3$-triggered release (Fig. 7.4). A similar potentiating effect on baseline ATP release has been demonstrated for the cytokine IFN-γ (Verderio *et al.*, 2001). It could be advocated that the largely elevated baseline level observed in the experiments presented in Fig. 7.4, attained a ceiling level impeding any further triggered release. However, the baseline elevation was much less pronounced in GP8 cells receiving 24h TNF-α treatment and also in the separate series of experiments shown in Fig. 7.5A. Under these less elevated baseline conditions, the net component of $InsP_3$-triggered release was still suppressed making the reaching of a ceiling, above which no further release is possible, an improbable explanation. At present it is not clear why the TNF-α-induced elevation of baseline ATP release was more prominent in the photolysis experiments (Fig. 7.4) as compared to the experiment with the zero calcium challenge (Fig. 7.5A). It might be related to different experimental conditions as the cells in the former case received electroporation treatment while those in the latter case did not. TNF-α also potentiated the baseline dye uptake into the cells and limited the triggered dye uptake to the baseline level (Fig. 7.5B). These results parallel the observations with ATP release and thus demonstrate similar TNF-α effects for the movement of a substance in the outside-in direction. Under these conditions, the reaching of a ceiling is very unlikely given the presence of the large extracellular dye pool available for moving into the cells. The experiments with gap 26, a blocker of $InsP_3$- and zero calcium-triggered connexin-dependent ATP release (Braet *et al.*, 2003b; Braet *et al.*, 2003c), demonstrate that the TNF-α induced elevation of basal ATP release is brought about by a non connexin-related release mechanism. TNF-α thus inhibits the net component of triggered ATP release and stimulates the baseline release by acting on two different ATP release mechanisms. In line with this notion, PKC activation with PMA, a known downstream signal of the TNF-α signaling cascade acting on connexins (Lampe *et al.*, 2000), did mimic the inhibitory effect of TNF-α on triggered ATP release but was without effects on baseline release. Taken over all, TNF-α appears to inhibit connexin-related pathways like gap junctional coupling and connexin hemichannel opening, while having opposite effects on non- connexin-related basal ATP release.

TNF-α thus seems to influence intercellular calcium signal communication in brain endothelial cells at several key points within the signaling cascade: first, it reduces gap junctional communication, and second, it inhibits paracrine purinergic communication by reducing the stimulated ATP release and possibly also by desensitizing purinergic receptors because of the increased basal release. Despite these actions, intercellular calcium wave propagation was only moderately inhibited (to 70-80 % of the control size). There are several possibilities to explain this apparent discrepancy: First, it is possible that other messengers such as ADP, UTP, glutamate or nitric oxide contribute to calcium wave propagation (Homolya *et al.*, 2000; Innocenti *et al.*, 2000; Lazarowski *et al.*, 1997; Moerenhout *et al.*, 2001; Willmott *et al.*, 2000a). Previous work indeed showed that a substantial part of the calcium wave remains after the combined inhibition of gap junctional and purinergic communication (Braet *et al.*, 2003b). Second, the presently used cell lines rely relatively more on gap junctions than on paracrine ATP signaling to communicate calcium signals (Braet *et al.*, 2003b), so complete block of triggered ATP release can translate into limited effects on calcium wave propagation. Finally, the size of calcium waves propagated through gap junctions does not lineary relate to the degree of cell-to-cell coupling (dye spread) as demonstrated by modeling studies (Sneyd *et al.*, 1995).

The combined effects of TNF-α on gap junctions and paracrine ATP signaling differ from the inverse relationship between gap junctional expression and purinergic calcium signaling that has been reported in astrocytes (Scemes, 2000), where a reduction of the former is associated with a stimulation of the latter (purinoceptor subtype shift). In brain endothelial cells, the actions of TNF-α seem to be targeted at depressing the two calcium signaling pathways. Depressed calcium signal communication can be hypothesized to reduce the spatial spread of blood-brain barrier opening, as lined out in the introduction. A possible interpretation could thus be that the cytokine limits the opening of the blood-brain barrier to the site where the lymphocytes, which initiate barrier opening, are transmigrating to leave the blood and enter the neural tissue.

Because TNF-α blocked the release step of paracrine purinergic signaling, it follows that all types of purinergic signaling, not only purinergic calcium signaling, will be affected. Reducing purinergic signaling at the blood-brain barrier might have profound effects on the function of the cells located in the vicinity of the barrier, i.e. the glial cells and the blood cells. Glial cells like astrocytes and microglial cells are indeed endowed with various purinoceptors that have been implicated in cell-to-cell calcium signal communication (Verderio *et al.*, 2001), in glutamate release and trophic effects in astrocytes (Fields *et al.*, 2000), and in the activation of microglial cells (Inoue, 2002). Potentially interesting is the fact that ATP can trigger TNF-α release from microglial cells (Inoue, 2002). If the inhibitory effect of TNF-α on triggered ATP release is also operative in microglial cells, ATP-triggered TNF-α release could feedback on these cells and thereby limit ATP release. Also of

potential interest is the fact that leukocytes and lymphocytes are endowed with purinergic receptors that mediate the proinflammatory actions of ATP on these cells (Di Virgilio *et al.*, 2001a; Di Virgilio *et al.*, 2001b). TNF-α is increased in neuroinflammatory diseases like multiple sclerosis and AIDS dementia (Navikas *et al.*, 1996), to concentrations compatible with the inhibitory effects observed in the present study (several tens of U/ml) (Sharief *et al.*, 1991). Decreased ATP signaling might thus be hypothesized to influence the complex immunological interactions of these blood cells with the blood-brain barrier endothelial cells. Further work will be needed to identify the mechanisms by which TNF-α exerts its effects on ATP release and to determine the functional impact of these actions.

chapter

8

Electroporation loading and photoactivation of caged $InsP_3$: tools to investigate the relation between cellular ATP release in response to intracellular $InsP_3$ elevation

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Abstract

Photolytic liberation of $InsP_3$ in single cells triggers cell-to-cell propagating calcium changes that are communicated by a gap junctional and a paracrine purinergic pathway involving $InsP_3$-triggered ATP release. We investigated the relation between the $InsP_3$ stimulus and the resulting ATP release in ECV304 cells using UV photolysis of caged compounds and bioluminescent ATP measurements. Careful consideration of all steps, starting from caged $InsP_3$ loading into the cells by electroporation, up to photoliberation upon UV exposure, allowed to derive a concentration-response relation that revealed a first part with a flattening ATP release response in the below 10 µM $InsP_3$ concentration range and a second phase of steeply increasing ATP release in response to above 10 µM $InsP_3$ stimulation. ATP release triggered by $InsP_3$ concentrations below 10 µM attained a level in the order of 30 % above baseline ATP release, while the steeply increasing response to high $InsP_3$ concentrations attained values in the order of 150 % above baseline. Our data indicate the involvement of both high affinity and low affinity $InsP_3$ receptors in the pathway leading to triggered ATP release, with activation of these receptors causing the release of 1-2 percent of the total cellular ATP pool.

8.1. Introduction

Photolytic liberation of messenger substances from inactive caged precursor molecules provides researchers with a powerful tool to study signal transduction pathways and their possible functional consequences (Adams *et al.*, 1993). Signal transduction pathways are especially prominent and diverse in the brain where a myriad of intracellular pathways and extracellular cell-to-cell signaling cascades are operating. Brain cells are equiped with a large variety of receptors that often show overlapping expression in different cells types such as neurons, astrocytes and vascular cells. Specific stimulation of a particular cell type by externally applying agonists selectively acting at plasma membrane receptors is therefore often difficult to achieve, unless the agonists are photoliberated extremely locally by two-photon photolysis (Pettit *et al.*, 1997). Targeted photolytic liberation of second messengers in the intracellular compartment of defined cells is another valuable approach to circumvent this problem, furthermore allowing in depth investigations of the downstream signaling mechanisms (Miyata *et al.*, 2000; Parpura *et al.*, 2000).

Previous work of our group has demonstrated that photoliberating $InsP_3$ in astrocytes and brain endothelial cells is an excellent tool to trigger calcium signals that are communicated between both cell types (Braet *et al.*, 2001; Leybaert *et al.*, 1998). $InsP_3$-triggered calcium signals can propagate between cells by the diffusion of a calcium messenger like $InsP_3$ via gap junctions and by the release of a purinergic messenger acting in a paracrine manner. In recent work we have demonstrated that photolytically elevating intracellular InsP3 triggers cellular ATP release that is, based on work with intracellular calcium buffers and certain connexin mimetic peptides, dependent on changes of cytoplasmic calcium and related to the presence of connexin-43 (Braet *et al.*, 2003b; Braet *et al.*, 2003c). These observations suggest that ATP release occurs via calcium-mediated opening of connexin hemichannels (connexin hemichannels are reviewed in Goodenough *et al.* (2003). The aim of the present work was to determine the relation between intracellular $InsP_3$ concentrations and the consequent cellular response of ATP release. In order to obtain an estimate of the $InsP_3$ concentrations attained upon photolysis, we determined the efficiency of loading cell-impermeable compounds like caged $InsP_3$ into a large number of cells and the efficiency of photolytic liberation. We furthermore describe these two methods in full detail. From these measurements we calculated that ATP release in response to $InsP_3$ is not saturating in the ten micromolar concentration range, suggesting the involvement of low affinity $InsP_3$ receptors. Calculations of the amount of ATP released by the cells in response to 10 µM $InsP_3$ suggest that a 1-2 % fraction of the total cellular ATP content is released upon a single $InsP_3$ pulse.

8.2. Materials and methods

8.2.1. Cell cultures and agents

In this study we used ECV304 cells (European Collection of Animal Cell Cultures, Salisbury, UK) grown on glass bottom microwells (MatTek Corporation, Ashwood, MA, US). Cultures were maintained in Medium-199 (Gibco, Merelbeke, Belgium) with 10 % fetal bovine serum (FBS) and with 2 mM glutamine until confluency. Experiments were done with the cells kept in Hanks' balanced salt solution buffered with 25 mM HEPES (HBSS-HEPES, pH 7.4). Adenosine 5'-triphosphate P3-1-(2-nitrophenyl)ethyl ester disodium salt (NPE-caged ATP) and D-myo-inositol 1,4,5-trisphosphate P4(5)-1-(2-nitrophenyl)ethyl ester trisodium salt (NPE-caged $InsP_3$) were obtained from Calbiochem (San Diego, CA). Dextran-tetramethylrhodamine (MW 10000), dextran-fluorescein (MW 70000), cascade blue hydrazide and fluo-3 AM were obtained from Molecular Probes (Leiden, The Netherlands). Probenecid was obtained from Sigma (Bornem, Belgium).

8.2.2. Electroporation

The cells were loaded with caged $InsP_3$ by electroporation. Electroporation loading was done on the microscope stage using a special electrode consisting of two parallel wires supported by a holder made from inert Delrin® acetal resin (Fig. 8.1). Cultures were briefly rinsed with a special electroporation buffer with low electrical conductivity (300 mM sorbitol, 4.2 mM KH_2PO_4, 10.8 mM K_2HPO_4, 1 mM $MgCl_2$ and 2 mM HEPES, pH 7.20) and thereafter a small volume (5 µl) of caged $InsP_3$ (200 µM) in electroporation buffer was added. The effective concentration of caged $InsP_3$ was approximately 100 µM because the added solution was diluted by a factor of about two because of the fluid remaining on the cells. Electroporation was done with a 50 kHz positive going signal applied in a pulsed manner as illustrated in Fig. 8.1C. After electroporation, cultures were washed with HBSS-HEPES and left 20 min before the start of the experiment. The electroporation solution also contained dextran-rhodamine (100 µM) to visualize the electroporation zone and to estimate the efficiency of the loading procedure. This was done by comparing the dextran-rhodamine fluorescence in the electroporation zone with the fluorescence of a thin layer of the same solution sandwiched between two coverslips. The thickness of the layer was approximately the thickness of the monolayer cell cultures (20-25 µm), as determined by inspection of fluo-3 loaded cultures with a confocal laser scanning microscope. The zone with caged $InsP_3$-loaded cells spreads out beyond the zone of electroporation loaded cells because caged $InsP_3$ (MW 635) is able to diffuse via gap junctions towards neighboring non-electroporated cells. The effective diffusion zone at a fixed time of 10 min after electroporation was determined to be 150 µm as

illustrated in Fig. 8.6. As a consequence an additional dilution factor of 800 (width of the electroporation zone in µm) divided by 800+150 (total width of caged $InsP_3$-loaded zone in µm) had to be taken into account in the calculation of the intracellular caged $InsP_3$ concentrations (dilution factor of 84 %).

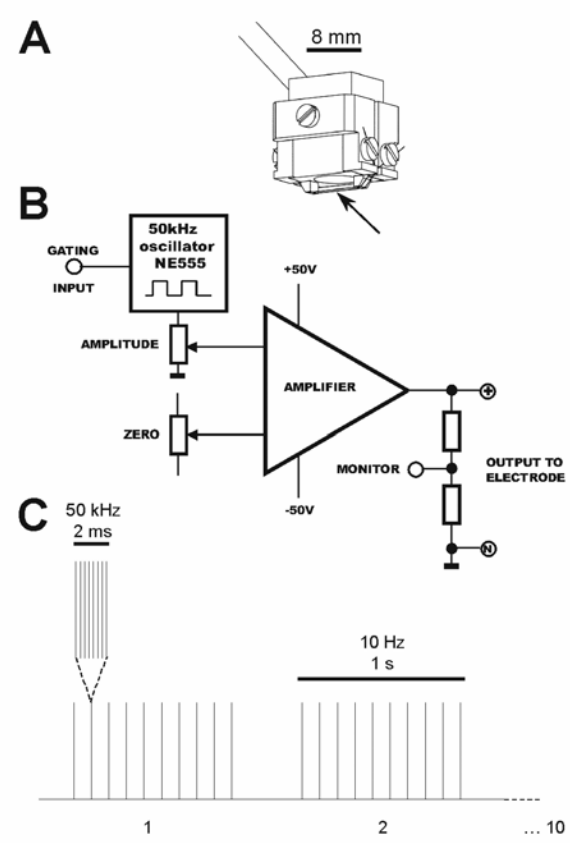


Fig. 8.1 *Electroporation setup to load the cells with caged $InsP_3$. A. The electroporation electrode consists of two parallel Pt-Ir wires (arrow) placed 400 µm apart (wire diameter 120 µm). The opening in the electrode holder (circular opening above wires) allows the passage of illumination light so that it can be positioned under microscopic control approximately 250 µm above the cells. B. Schematic drawing of the electrode driver circuit. The signal from a 50 kHz oscillator circuit (NE555 astable oscillator) is amplified to a signal amplitude that can be adjusted between 0 and 50 V. The gating input receives a TTL signal from a stimulator unit to produce repeated trains of 50 kHz pulses. C. The pulse train protocol used in the experiments. The voltage amplitude was 20 V giving an electric field strength of 500 V/cm (wire separation 400 µm).*

8.2.3. UV illumination

UV spot illumination was performed to trigger intercellular calcium waves in response to photolytic liberation of caged $InsP_3$, using the setup illustrated in Fig. 8.2A. BS1 in this figure was a 70 % transmission - 30 % reflection mirror and BS2 a 30 % transmission-70 % reflection mirror (XF122 and XF123 resp., Omega Optical, Brattleboro, VT). The optic fiber was a single mode fiber with a diameter of 200 µm; in combination with a x40 objective lens (Nikon S fluor 40x NA 1.3 oil) this gives a spot size of 5 µm diameter. The UV light source was either a flash lamp system (type JML-C2, Rapp OptoElectronic, Hamburg, Germany) or the 351 nm UV line from an argon laser (type 2018, Spectra-Physics, Eindhoven, The Netherlands). All experiments were performed on Nikon Eclipse TE300 microscopes. Other valuable alternatives for spot illumination are discussed in Parpura *et al.* (1999b).

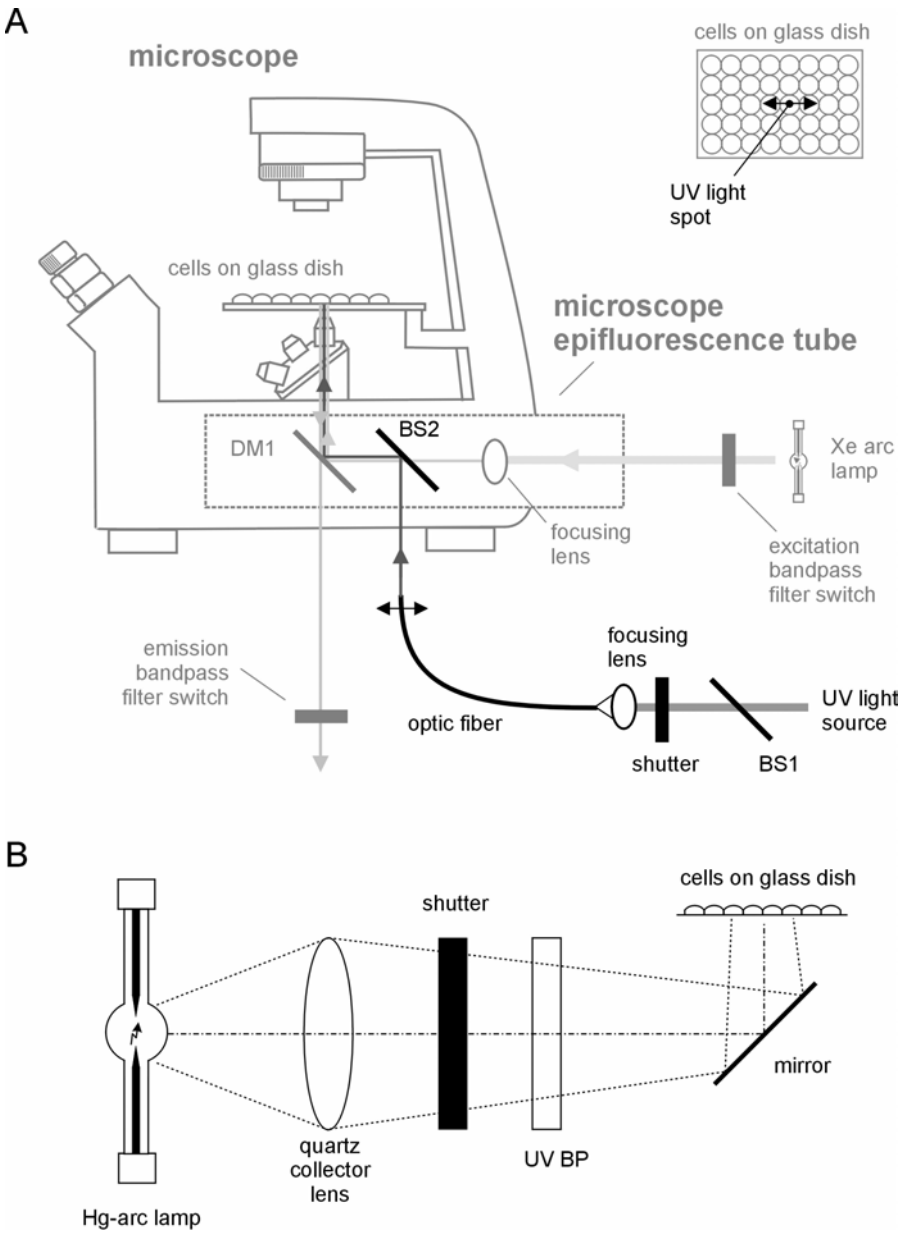


Fig. 8.2 *Optical setup for delivering UV light to a single cell (UV spot illumination) or to multiple cells (UV field illumination). A. UVspot illumination. UV light from a laser source is coupled to a single mode optic fiber by means of a focusing lens. The UV light traverses a beam splitter (BS1). The microscope epifluorescence tube is adapted such that a second beamsplitter (BS2) is inserted close to the dichroic mirror in the microscope (DM1). The aperture of the optic fiber is positioned to form a focused image at the level of the cells on the glass dish. In this way, the fiber aperture appears as a spot (UV light spot) with a size inversely proportional to the magnification factor of the objective lens. The position of the spot in the cell field can be changed (bidirectional arrow on glass dish) by moving the fiber aperture laterally (bidirectional arrow at optic fiber). The duration of the UV exposure is determined by the shutter. The epifluorescence excitation light and the fluorescent light pathways illustrate the standard epifluorescence configuration. B. UV field illumination. A focused image of the light arc of a Hg-arc lamp is formed at the level of the cells on a glass dish by means of a quartz collector lens. The light passes an electronically controlled shutter and a UV bandpass filter (UV BP).*

UV field illumination was used to photorelease $InsP_3$ in a large zone of caged $InsP_3$-loaded cells, in order to obtain a measurable amount of ATP released by the cells in response to this stimulus. Light from a 100 W Hg-arc lamp with quartz collector lens (LH-M100C-1, Nikon) was bandpass-filtered at 330 nm (330WB80, Omega Optical, Brattleboro, VT), reflected by a mirror (10D510AL.2, Newport, Leuven, Belgium) and focused to the culture thereby exposing a large zone of cells (Fig. 8.2B). The energy of the photolytic UV light striking the cells was varied by changing the exposure time by means of a shutter. The energy density at 5 s exposure time was calculated from power measurements with a UV power meter (model 1830-C with 818-UV detector, Newport, Leuven, Belgium) to be in the order of 500 mJ/cm². The efficiency of photoliberating caged compounds by field illumination was investigated by exposing a thin layer of caged ATP (1 µM) to the photolytic UV light and measuring the amount of photoliberated ATP with a bioluminescent ATP assay kit (see ATP measurements). The attenuation of the photolytic UV light by its passage through the cell monolayer (reduction to 72 %, see Results) was determined with UV power measurements under conditions with and without cells. Taking into account this attenuation, a 2 s UV exposure time corresponded to 57 % photolytic efficiency for uncaging $InsP_3$ in the cells' interior. Control experiments on cultures loaded with vehicle alone showed that UV illumination by itself did not trigger any calcium wave or ATP release.

8.2.4. Calcium imaging

Intercellular calcium waves were monitored with epifluorescence microscopy and digital imaging using the calcium-sensitive dye fluo-3. Cell cultures were loaded by incubation for 1h at room temperature in HBSS-HEPES containing 10 µM fluo 3-AM and 1 mM probenecid. Cultures were then washed with HBSS-HEPES and left at room temperature for 30 min for de-esterification. Images were captured using an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, UK) and transferred directly to a PC equipped with an image acquisition and processing board (DT3155, Data Translation, Marlboro, MA). Calcium changes were expressed as relative fluorescence changes, i.e. $\Delta F/F_0 = [F_t - F_0] / F_0$ (with F_0 the fluorescence before stimulation and F_t the time-dependent fluorescence signal after stimulation). The extent of cell-to-cell propagation of calcium changes was determined from the size of the intercellular calcium wave at its maximal state of extension and was quantified by determining the surface area where $\Delta F/F_0$ was above a threshold of 50 %. The surface area is a more sensitive parameter than the wave radius to detect changes in the extent of calcium signal propagation because changes are amplified quadratically as compared to the radius. $\Delta F/F_0$ and calcium wave size calculations were done with the custom developed program FluoFrames (available upon request).

8.2.5. ATP measurements

ATP release was determined using a bioluminescent ATP assay kit (product no. FL-AA, Sigma). In the experiments using caged ATP, a thin layer of solution was exposed to UV light and immediately thereafter mixed with the same quantity of ATP assay mix solution. In the experiments probing cellular ATP release in response to $InsP_3$ photoliberation, 100 µL of 200 µL supernatant was collected immediately (within 5 s) after UV exposure and transferred to 100 µL ATP assay mix solution (used at 5-fold dilution). The mixture was placed in a sample holder of a custom build luminometer and light emission was measured by photon counting with a photomultiplier tube (9924B, Thorn-Emi Electron Tubes, Middlesex, UK). ATP calibration curves were performed in a concentration range of 50-1000 nM. The ATP results presented in the text as the fractional release of the total cellular ATP content were calculated taking into account a caged $InsP_3$-loaded cell surface area of 7.6 mm² (8 mm x 0.95 mm), a cell density of 1140 $\pm$ 200 cells/mm² (n=4) and a cellular protein content of 8.3 $\pm$ 1.4 ng protein per cell (n=4). A slightly different calculation was done for control purposes and was based on a 5 mM intracellular ATP concentration (Allue *et al.*, 1996) and a spheric cell geometry with an average radius of 15 µm. We also took into account the fact that the released ATP will diffuse and act on cells in the vicinity that were not directly photostimulated with $InsP_3$ but that will release ATP upon activation of purinergic receptors (Cotrina *et al.*, 1998a). A 10 s diffusion time between the start of photostimulation and collection of the supernatant (2s + 5s, rounded) allows half of the released ATP to diffuse over a distance in the order of 60 µm (ATP diffusion constant of 0.35 10^{-5} cm²/s; Hubley *et al.*, 1995). The zone of effectively ATP releasing cells was therefore taken as 60 µm beyond the borders of the strip-like $InsP_3$ stimulated zone (8 mm x 0.95 mm), giving a surface area 8.6 mm² (8 mm x 1.07 mm).

8.2.6. Data analysis and statistics

The data are expressed as mean $\pm$ SEM with n denoting the number of experiments; SEM is not depicted for datapoints with n = 2. Statistical significance was assessed by the Student's *t*-test for unpaired observations, with a p value below 0.05 indicating significance. All curve fittings were done to a hyperbolic saturating function using Levenberg-Marquardt least squares routines; the dashed line part in Fig. 8.9 represents an arbitrary function.

8.3. Results

8.3.1. Efficiency of caged $InsP_3$ loading

Photolytic liberation of $InsP_3$ from caged $InsP_3$ is an elegant manner to trigger calcium signals that propagate over several cell layers as intercellular calcium waves and to induce the release of ATP from the cells (Braet *et al.*, 2001; Braet *et al.*, 2003b; Braet *et al.*, 2003c). Caged $InsP_3$ is a charged molecule that can be loaded into monolayer cell cultures by electroporation. This involves placement of an appropriate electrode close to the cells and application of a pulsed radio-frequency electric field (Fig. 8.1). Electroporation is done with the cells bathed in a special medium containing caged $InsP_3$ and a fluorescent reporter dye. The reporter dye (dextran rhodamine 10 kDa) is included to visualize the zone of electroporated cells and to evaluate the efficiency of the loading. The loading efficiency decreased with increasing molecular weight of the substance to be loaded and was expected to be in the order of 25 % for caged $InsP_3$ loaded into ECV304 cells (Fig. 8.3).

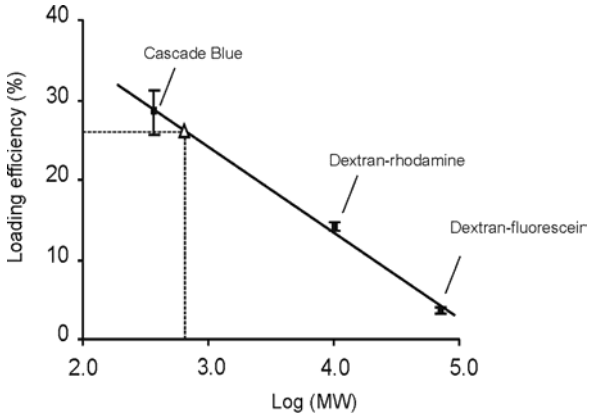


Fig. 8.3 *Efficiency of electroporation loading in function of the molecular weight. Fluorescent dyes with various molecular weights were added to the electroporation medium and the resulting fluorescence in ECV304 cells was quantified relative to the fluorescence of a thin layer (thickness of one cell layer) of the same dye solution. The loading efficiency decreased with increasing molecular weight. The triangle indicates the position of caged $InsP_3$ on this plot and suggests a loading efficiency slightly above 25 %. Data derived from 3 experiments.*

The loading efficiency also varied with the location of the cells under the electrode and was higher at the neutral electrode wire as compared to the positive electrode wire (Fig. 8.4A). This was reflected by the size of intercellular calcium waves, triggered by exposure of a single caged $InsP_3$-loaded cell to a small spot of photolytic UV light (Fig. 8.5A); the calcium wave size elicited in this way was larger in cells located under the neutral electrode wire and thus paralleled the difference in loading efficiency (Fig. 8.4B).

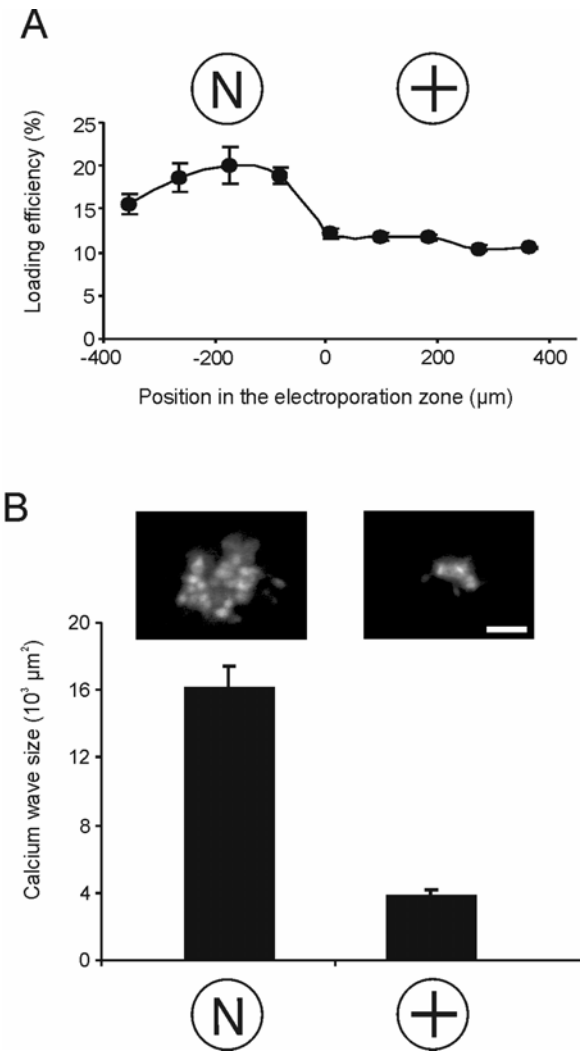


Fig. 8.4 *Loading efficiency profile in the electroporation zone (ECV304 cells). A. The loading was not homogenous in the zone directly situated under the electrodes and was more prominent under the neutral pole electrode (wire marked N) as determined from the fluorescence of the dextran rhodamine reporter dye. B. This was also apparent from the size of intercellular calcium waves triggered by photoliberation of $InsP_3$ by exposure of single cells (loaded with caged $InsP_3$) to UV spot illumination. The two images on top of each bar illustrate representative calcium waves at their maximal extent of propagation. The white calibration bar measures 50 µm. Data derived from 14 experiments.*

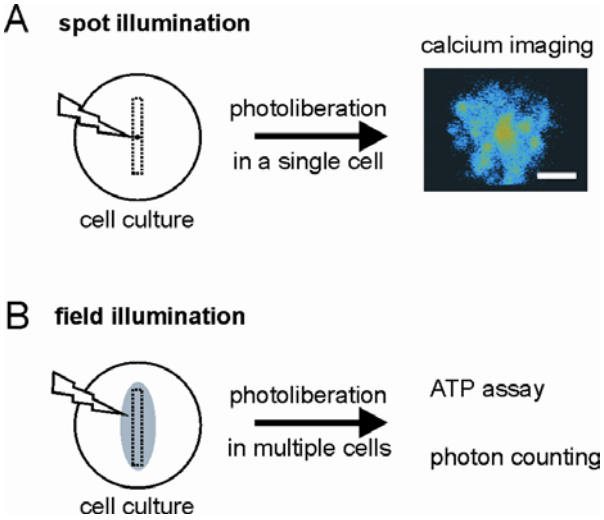


Fig. 8.5 *Experimental setup for photoliberation of $InsP_3$ in single or multiple cells. A. Cell cultures loaded with caged $InsP_3$ by electroporation (rectangular zone delineated by the dashed line) were exposed to spot UV illumination (black dot in the middle of the cell culture) directed to a single cell to trigger calcium changes that propagate from te stimulated cell to neighboring cells as an intercellular calciul wave (image at the right). B. Exposure of caged $InsP_3$-loaded cell cultures (rectangular zone as in A) to field UV illumination (gray zone) was used to measure ATP release by the cells in response to this $InsP_3$ stimulus.*

Caged $InsP_3$ (MW 635) is a molecule that can diffuse through gap junctions (Leybaert *et al.*, 1998) and the zone of cells loaded with this substance can therefore extend beyond the borders of the electroporation zone indicated by the reporter dye (MW 10 kDa, not gap junction permeable). To determine the exact borders of the cell zone loaded with caged $InsP_3$, we applied the UV spot stimulus to cells beyond the border of the electroporation zone and recorded the resulting intercellular calcium wave (at a fixed time after electroporation). The size of the intercellular calcium wave declined with the distance from the border (Fig. 8.6). Taking into account this gap junctional spread, the total width of the caged $InsP_3$-loaded cell zone was in the order of 950 µm (800 µm for the zone indicated by dextran rhodamine plus a diffusion zone of 150 µm at the side of the neutral electrode).

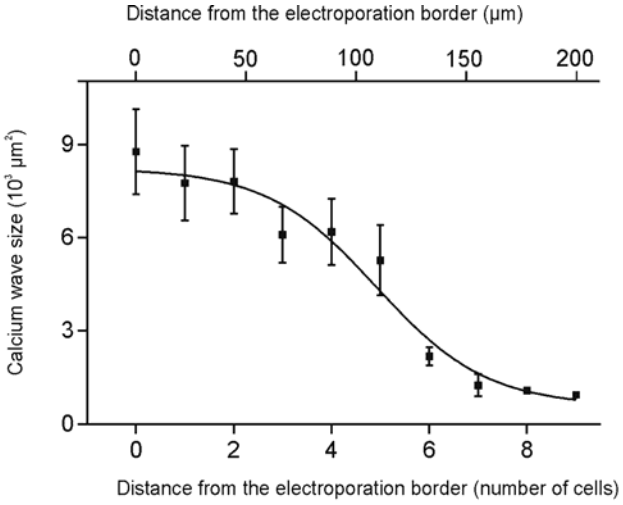


Fig. 8.6 *Profile of caged $InsP_3$ loading away from the electroporation zone. In this experiment intercellular calcium waves were triggered by UV spot illumination directed to single cells away from the electroporation border, at a fixed time after electroporation. The abscissae express the distance from the border of the electroporation zone, identified by the dextran rhodamine reporter dye. Intercellular calcium waves could be elicited from cells away from the electroporation border because caged $InsP_3$ diffuses from electroporation-loaded to non-loaded cells via gap junctions. The calcium wave size decreased with the distance from the border. The zone of caged $InsP_3$-loaded cells is thus larger than the zone indicated by the dextran coupled reporter dye. Data from at least 4 experiments.*

8.3.2. Efficiency of photoliberation

To determine the intracellular $InsP_3$ concentrations attained by whole field photolysis of caged $InsP_3$ (described below), we established the efficiency of the photolytic process, i.e. the relative amount of $InsP_3$ liberated from its caged precursor. These measurements were performed with caged ATP because the liberated amount of ATP can be easily determined with the luciferin-

luciferase assay. The photolytic efficiency increased with increasing UV exposure times, i.e. increasing UV energies, followed by a flat trajectory where the response attained saturation (Fig. 8.7). Because caged ATP and caged $InsP_3$ have comparable quantum yields (0.63 and 0.65 respectively), the photolytic efficiency curve obtained with caged ATP also applies for caged $InsP_3$. However, caged $InsP_3$ was used in conjunction with cell cultures that will attenuate the intensity of the photolytic UV light. The UV light passing through monolayer cell cultures was reduced to 72 ± 5 % (n=5) of the power passing through a layer of equal thickness of HBSS solution only. The exposure times depicted in Fig. 8.7 should be multiplied by the same factor (0.72) to convert this graph to a photolytic efficiency curve for uncaging intracellulary located caged $InsP_3$.

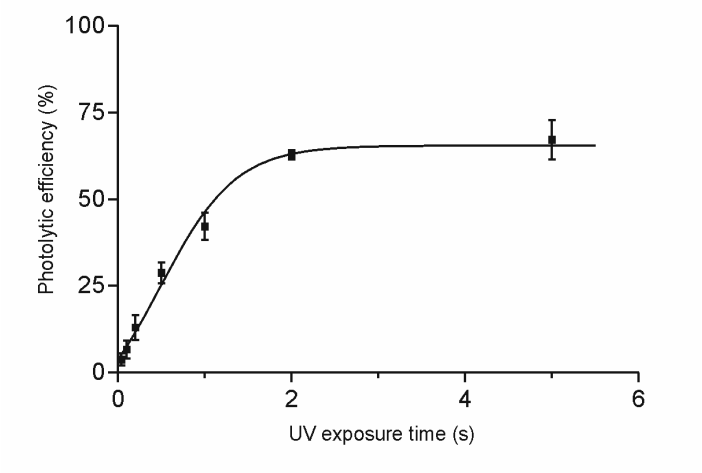


Fig. 8.7 *Photolytic efficiency in function of the UV exposure time. A thin layer of caged ATP was exposed to UV field illumination and the concentration of photoliberated ATP measured with a luciferin-luciferase assay. The ordinate expresses the concentration of photoliberated ATP relative to the concentration of caged ATP present in the solution, thus giving an indication of the efficiency of the photolytic process. The graph illustrates that the photolytic efficiency, and thus the amount of probe liberated upon uncaging, increases with the duration of UV exposure until saturating is attained. Data from 4 experiments.*

8.3.3. Dose-response relation between UV exposure and cellular ATP release

Intercellular calcium waves triggered by photolytic liberation of $InsP_3$ in a single cell are communicated to neigboring cells by the diffusion of $InsP_3$ through gap junctions and by a paracrine pathway involving the release of ATP from the cells (Braet et al., 2001). We have applied the $InsP_3$ stimulus to a large area of cells in order to document cellular ATP release (Braet et al., 2003b; Braet et al., 2003c). Investigating the relation between the duration of exposure to photolytic UV light and the amount of ATP released by the cells, showed that ATP release increased with longer UV exposures and thus with the amount of photoliberated $InsP_3$. The slope of this relation flattened with increasing stimulus strength, without however reaching full saturation within the range of stimuli applied (Fig.8.8). A similary shaped graph was obtained in previous work

for the relation between UV exposure of single caged $InsP_3$-loaded cells and the size of the consequently triggered intercellular calcium wave (Leybaert et al., 2000). Comparison of the photolytic efficiency curve (Fig. 8.7) with the cellular ATP release curve (Fig. 8.8), taking into account the correction for the presence of cells, reveals that ATP release continues to increase in a range of flattening photorelease efficiency and thus of flattening $InsP_3$ concentrations. This indicates that ATP release is very sensitive to small $InsP_3$ changes in this range of stimulus strengths.

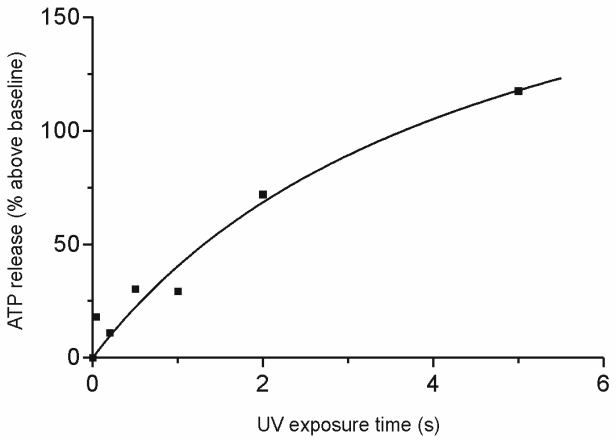


Fig. 8.8 *Dose-response relation between UV exposure time and the concentration of $InsP_3$-triggered ATP release in ECV304 cells. In this experiment, cells were electroporation-loaded with caged $InsP_3$ and exposed to UV field illumination during the various times indicated. The concentration of ATP released by the cells in response to $InsP_3$ was determined with a luciferin-luciferase assay and expressed relative to the baseline ATP release. The curve flattened at higher UV exposure times but did not reach full saturation. Data from at least 2 experiments.*

8.3.4. Concentrations of photoliberated $InsP_3$ and cellular ATP release

To estimate the $InsP_3$ concentrations that are reached following photoliberation, we took into account the various correction and dilution factors and the efficiencies of electroporation loading and photoliberation. Starting from a 100 µM caged $InsP_3$ concentration in the loading medium, the concentration of $InsP_3$ photoliberated by 2 s UV exposure was calculated to be in the order of 12 µM (note that all $InsP_3$ concentrations given are only estimates). Similar calculations for all exposure times used, makes it possible to construct a graph expressing ATP release in function of the $InsP_3$ concentration (Fig. 8.9). This graph illustrates a small and saturating ATP release level in response to low $InsP_3$ concentrations and a sharp increase of ATP release for above 10 µM $InsP_3$ concentrations, indicating that the ATP release process is indeed very sensitive to $InsP_3$ concentration changes in this particular concentration range. With the 2 s – 12 µM $InsP_3$ stimulus setting, the net ATP concentrations released by the cells in 200 µL medium amounted to 82 ± 25

nM (n=16). Expressed relative to the cellular protein content, the ATP released by the $InsP_3$-stimulated cells, and also by the neigboring cells activated by ATP diffusing in from the directly stimulated zone, averaged 202 pmol per mg of cell protein. Comparing this value to the published intracellular ATP content in these cells of 20 nmol per mg of cell protein (Hurst *et al.*, 2001) indicates that approximately 1 % of the total cellular ATP content was released into the extracellular space in response to photolytic stimulation with 12 µM $InsP_3$. Slightly different calculations based on a 5 mM cellular ATP content (Allue *et al.*, 1996) indicated a value of approximately 2.5 % of the total cellular ATP content being released. A fraction of 1 - 2.5 % of a total ATP content of 100 - 200 fmol per cell means that femtomoles of ATP are released upon a single 12 µM $InsP_3$ stimulus.

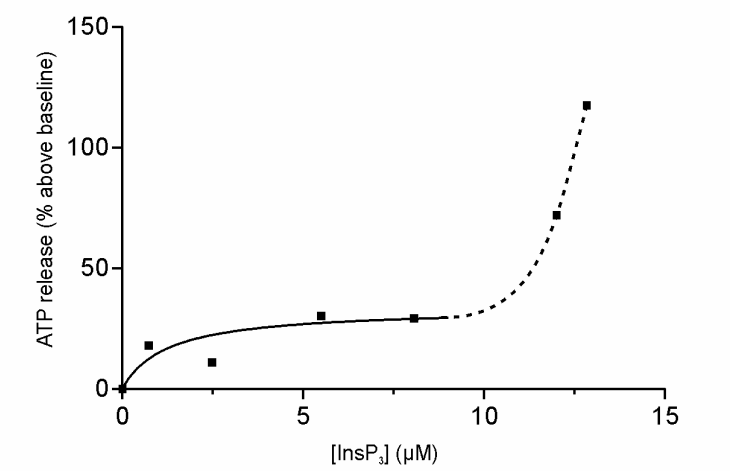


Fig. 8.9 *Concentration-response relation between the $InsP_3$ concentration liberated upon photolysis and the ATP released in response to this stimulus. The graph is essentially the same as presented in Fig. 8.5 but the UV exposure times have been transformed to $InsP_3$ concentrations based on the various correction factors, dilution factors and efficiencies discussed in the text. The data points indicate a bimodal response shape, displaying saturating ATP release at low $InsP_3$ concentrations and a steeply increasing ATP release at concentrations above 10 µM.*

8.4. Discussion

The present work was undertaken to obtain information on the concentrations of $InsP_3$ needed to trigger cellular ATP release and the amounts of ATP released. By considering all steps involved in the loading and photocleavage process, we obtained a concentration-response relation characterized by a low degree of ATP release, attaining a level approximately 30 % above basal release, with below 10 µM $InsP_3$ concentrations and a second phase of increasing ATP release with $InsP_3$ levels above 10 µM. The fractional release appeared to be quite a significant portion of the cellular ATP content.

The work was performed on ECV304 cells because these cells display a significant degree of ATP release in response to photoliberated $InsP_3$ (Braet *et al.*, 2003b). $InsP_3$-triggered ATP release in these cells is calcium-dependent (based on the inhibitory effects of intracellulary applied BAPTA). $InsP_3$-triggered ATP release is also potently blocked by connexin mimetic peptides like gap 26 or gap 27, the action of which seems to critically depend on the presence of connexin-43 in the cells (Braet *et al.*, 2003b). $InsP_3$-triggered ATP release in ECV304 cells thus concerns an intruiging release pathway that possibly involves calcium-mediated opening of connexin hemichannels. Up to now we have not been able to demonstrate significant ATP release in response to photolytically increasing intracellular calcium (Braet *et al.*, 2003b), precluding investigations of the dose-response relation between calcium and connexin-related ATP release. The present study was therefore directed more upstream to the relation between $InsP_3$ and ATP release.

In order to estimate the amounts of $InsP_3$ released upon UV exposure, we determined a dose-response relation between applied UV energy (determined by the UV exposure time) and the photolytic efficiency of uncaging ATP from caged ATP, a probe with similar quantum yield as $InsP_3$. Other methods are available to determine the amount of photoreleased substance, such as monitoring pH changes induced by protons released with uncaging (Khodakhah *et al.*, 1993) or HPLC (Walker *et al.*, 1988), but the presently used method is interesting in that ATP photoreleased from caged ATP can be measured directly with a very sensitive bioluminescent assay. We took into account all possible corrections necessary to make the data obtained with caged ATP applicable to caged $InsP_3$. We varied the exposure time to the photolytic light in order to attain different $InsP_3$ concentrations necessary to construct the dose-response relation. As a consequence, the large $InsP_3$ concentrations are only attained after a build-up period where desensitization effects might occur. Prolonged increases of $InsP_3$, by activating the $Gq\alpha$ or PLC pathway over several hours, has been described to desensitize $InsP_3$ receptors (Honda *et al.*, 2001; Wojcikiewicz *et al.*, 1994) but seconds $InsP_3$ exposure times, as applied in the present work, have no desensitizing effects (Oldershaw *et al.*, 1992). Another point concerning seconds lasting $InsP_3$ elevations, relates to the degradation of $InsP_3$ by specific phosphatases: caged $InsP_3$ is not

affected by these phosphatases (Walker *et al.*, 1987), but the attained $InsP_3$ concentrations presented might be slightly overestimated because of InsP3 breakdown during prolonged UV exposure. Taking into account an $InsP_3$ degradation rate of 155 pmoles/min per mg of cell protein (rate for 12 µM $InsP_3$, calculated from Qazi *et al.* (1999) reduces the $InsP_3$ level attained with 2 s photolysis to about 75 % (9 µM) of its estimated 12 µM value. With this correction, the $InsP_3$ threshold for steeply increasing ATP release thus remains in the 10 µM range.

Our data demonstrate a biphasic ATP release pattern in response to increasing $InsP_3$ concentrations and thus points towards the involvement of both high and low affinity $InsP_3$ receptors in the cascade leading to triggered ATP release. $InsP_3$ receptors found in various tissues are the well known type 1, 2 & 3 receptors that are found in various tissues and that are characterized by Kd's for $InsP_3$ binding in the range of 1-50 nM (Maeda *et al.*, 1990; Mignery *et al.*, 1989; Ross *et al.*, 1989). ECV304 cells are cells of vascular endothelial origin that are known to possess both type 1 and type 3 receptors (Mountian *et al.*, 1999). Functional studies of type-1 and type-3 receptors, in contrast to binding studies, indicate EC_{50}'s in the order of several micromolar(Kaftan *et al.*, 1997; Thrower *et al.*, 2001), and responses have been reported with $InsP_3$ values going as high as 80 µM (Khodakhah *et al.*, 1993) for the type-1 receptor while EC50 values of 18.6 µM have been reported for the type 3 receptor (Miyakawa *et al.*, 1999). The biphasic ATP release pattern in response to increasing $InsP_3$ concentrations observed in the present study thus probably reflects the activation of various $InsP_3$ receptor isoforms present in ECV304 cells.

The levels of cellular ATP release in response to multimicromolar intracellular $InsP_3$ apparently involves quite a significant fraction of the total cellular ATP content, and repetition of tens of such stimuli will be expected to deplete cells from their most important energy substrate. ATP release with below 10 µM $InsP_3$ stimuli gives fractional release values that are 2-3 times smaller, coming more close to the 0.1 % fractional release that has been reported in renal epithelial cells in response to hypotonicity (Schwiebert, 2001). ATP release by endothelial cells is an essential element of the important paracrine communication pathway from the bloodvessels towards the blood cells. Endothelial ATP has for example been demonstrated to act as a proinflammatory signal on blood leukocytes and lymphocytes (Di Virgilio *et al.*, 2001a; Di Virgilio *et al.*, 2001b). The large amounts of ATP released by endothelial cells in response to above 10 µM $InsP_3$ concentrations can thus be hypothesized to be necessary to overcome dilution and washout of the endothelial ATP signal by the bloodflow. In conclusion, ECV304 cells demonstrate functional responses to high $InsP_3$ concentrations applied photolytically, by releasing ATP into the extracellular space. The steep concentration dependence of this response suggests the involvement of low affinity $InsP_3$ receptor sites in the signaling cascade leading to ATP release.

chapter

9

General discussion and perspectives for future research

9.1. General discussion

The communication between cells is a key facet of every biological system. Intercellular communication is vital to the proper functioning of a complex multicellular network, such as the brain. Considerable progress has been made in recent years in clarifying the subtleties of intercellular communication. Numerous signaling molecules have been identified and mechanisms have been unraveled.

In the brain, capillary endothelial cells are ensheated by astrocytic foot processes, allowing them to exchange signals. In addition to this anatomical basis for a signaling system between both cell types, functional evidence for a fast astrocyte-endothelial signaling pathway was suggested by the discovery that astrocytes as well as endothelial cells exhibit a form of excitability, namely calcium excitability (Vesce *et al.*, 2001). Calcium excitability shows some similarities with the electrical excitability of neurons, and denotes the fact that astrocytes and endothelial cells react to a calcium stimulus with a calcium-induced calcium release response acting to amplify the signal and allowing the communication of the signal to neighboring cells.

The initial idea of this work was to determine how calcium signals, brought about in astrocytes by neuronal activity, can be communicated further along this pathway towards the blood vessels, more specifically towards the endothelial cells. Our work, performed in astrocyte and endothelial cultures or co-cultures, making use of microscope calcium imaging, photoactivation techniques and ATP release measurements demonstrates that:

1. Calcium signals are communicated between astrocytes and endothelial cells in a bidirectional way by the diffusion of $InsP_3$ through gap junction channels and by the release of a purinergic messenger acting on neighboring cells. The contribution of both pathways depends on the triggering stimulus used.

2. Endothelial calcium signal communication also occurs through these two pathways, with some cell lines displaying a clear and prominent component of cellular ATP release triggered in response to cytoplasmic $InsP_3$ elevation. This triggered ATP release is calcium-dependent and involves, based on work with a connexin mimetic peptide (gap 26), a connexin-related mechanism.

3. Cellular ATP release, triggered by intracellular $InsP_3$ or by zero extracellular calcium, is inhibited by connexin mimetic peptides (gap 26, gap 27), by gap junction blockers and by trivalent ions. Connexin mimetic peptides only display their inhibitory effect when the appropriate connexins are present. Uptake of a reporter dye into the cells, triggered by zero calcium exposure displays a similar pharmacological sensitivity. Short exposure to

connexin mimetic peptides does not affect gap junctional coupling.

4. TNF-α inhibits calcium signal communication between endothelial cells by reducing gap junctional coupling, by increasing basal ATP release and by blocking the triggered component of ATP release. It appears to inhibit two connexin-related pathways: gap junction channels and connexin hemichannels.

5. $InsP_3$-triggered ATP release displays a concentration-response relation that does not saturate in the 10 µM range indicating interaction with low affinity $InsP_3$ receptors.

What are the mechanisms of astrocyte-endothelial calcium signal communication?

In chapter 4, intercellular calcium signals were triggered in astrocyte-endothelial co-cultures by either gentle mechanical stimulation or photoliberation of $InsP_3$. Mechanical stimulation clearly evoked bidirectional calcium waves that were carried by both the diffusion of $InsP_3$ through gap junction channels (the gap junctional pathway) and by the release of a purinergic messenger acting on neighboring cells (the paracrine purinergic pathway). In astrocytes, the $InsP_3$ stimulus apparently triggered the gap junctional pathway in isolation, without invoking the extracellular purinergic pathway. By contrast, the endothelial cells solicited both pathways. This is probably the reason why endothelial $InsP_3$-triggered calcium signals propagated more extensively into the astrocytes as compared to astrocytic calcium signal propagation in endothelial cells. In addition, there are some alternative explications for this directional difference, including rectifying gap junctions, or different amounts of $InsP_3$ loaded into these two cell types because of morphological differences in geometry.

An important point concerns the question how far the co-culture model represents the situation *in vivo*. A controversial point is for example the presence of gap junctions between astrocytes and endothelial cells. There is currently no morphological evidence for such junctions but functional data in the *in vivo* or acute brain slice preparation are still missing. One of the important findings of our work is that poorly coupled cells, such as astrocytes and endothelial cells, are still able to communicate calcium signals through the gap junctional pathway. Even small amounts of $InsP_3$ can trigger measurable calcium transients because calcium-induced calcium release amplifies the initial small amplitude calcium signals.

Our work illustrates a fast acting (time scale of seconds) communication pathway between astrocytes and endothelial cells, which might form a bi-directional communication pathway transferring either blood-borne signals to the neuropil, thereby affecting neuronal and synaptic functioning, or carrying astrocytic signals to the blood-brain barrier, thereby modulating its transport

and barrier functions. Endothelial calcium signals are central to the control of the paracellular permeability of the blood-brain barrier but there is no evidence that this kind of barrier opening occurs under physiological conditions. Our hypothesis for further work is rather that endothelial calcium signals modulate the specific transport proteins acting to shuttle important substrates like glucose over the barrier, as reported by Mitani *et al.* (1995) be it in completely different cell types. In preliminary work we have demonstrated that agonists, acting primarily on cytoplasmic calcium, are able to stimulate the transport of glucose via the GLUT-1 transporter (Braet *et al.*, 2000a). In this context we propose that there is, in addition to the well known neurovascular and neurometabolic coupling phenomena in the brain (Magistretti *et al.*, 1999b; Zonta *et al.*, 2003) also coupling of neuronal activity to the barrier, to increase the transport of glucose in order to accommodate the temporary increased energy needs of the neural tissue. Further work of the research group is directed towards documenting this hypothesis.

What is the role of $InsP_3$ in paracrine purinergic signaling and is it involved in triggering cellular ATP release?

A possible explanation for the more extensive calcium signal propagation from endothelial cells to astrocytes as compared to the opposite direction, is that endothelial cells upon activation invoke both the gap junctional and the paracrine purinergic pathways. We therefore further investigated the contribution of these two pathways in various endothelial cell lines in more detail (chapter 5). All endothelial cell lines tested, used both the intra- and the extracellular signaling pathway to propagate $InsP_3$-triggered calcium signals through the culture (Fig. 9.1), with a different relative contribution of each of these pathways. This difference is most likely related to differences in the expression levels of connexins, proteins involved in extracellular messenger release and receptors characteristic of the two pathways.

The paracrine purinergic component was most prominent in ECV304 cells and we therefore used these cells to directly document that stimulation with $InsP_3$ triggers ATP release. $InsP_3$ indeed triggered cellular ATP release and the release process appeared to be dependent on calcium (experiments with intracellular BAPTA), although increasing calcium by itself was not able to trigger ATP release. This finding is in line with the fact that we have never been able to demonstrate cell-to-cell communication of calcium signals in response to an increase of cytoplasmic calcium. This is obvious for the gap junctional pathway because, although both calcium and $InsP_3$ can diffuse through gap junctions, $InsP_3$ diffuses faster in the cytoplasm because it does not bind to immobile buffers as does calcium. It is however less clear why an increase of calcium, of a magnitude similar as the one brought about by $InsP_3$, was not able to trigger cellular ATP release. Presumably, photoliberation of calcium from the caged calcium compound NP-EGTA brings about a global

calcium increase in the cytoplasm that does not attain a sufficient amplitude at the target site, while $InsP_3$ triggers calcium release form the stores, i.e. from confined site close enough to the calcium-dependent release machinery.

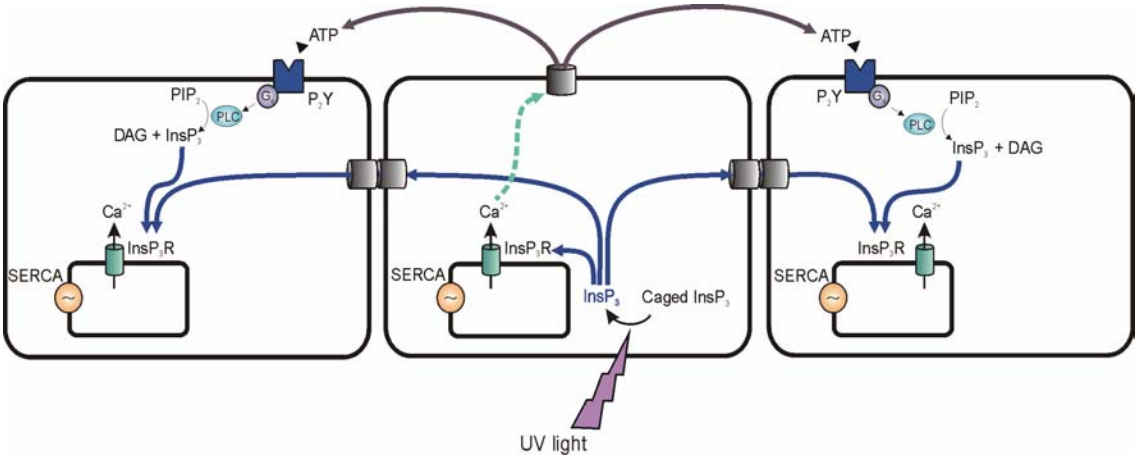
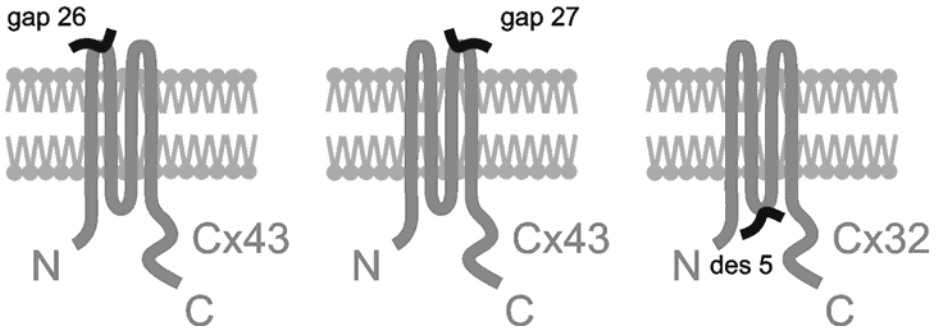


Fig. 9.1 $InsP_3$-triggered calcium signal in a single endothelial cell propagates to neighboring endothelial cells. Upon photocleavage of caged $InsP_3$, the second messenger $InsP_3$ suddenly becomes available to bind its receptor on the calcium stores and release calcium into the cytosol. The calcium signal propagates via an intracellular pathway involving $InsP_3$ diffusion through gap junctions and calcium release from stores in neighboring cells, and an extracellular (paracrine) pathway involving the release of a purinergic messenger like ATP diffusing in the extracellular space and acting on purinergic receptors on neigboring cells. Our work with connexin mimetic peptides indicates that ATP release occurs through connexin hemichannels, i.e. half gap junction channels not connecting to their homologues on neigboring cells.

We applied the connexin mimetic peptide gap 26 to investigate the nature of $InsP_3$-triggered ATP release. The main hypothesis behind this work was that if ATP release occurs through connexin hemichannels, these peptides which have, in hemichannels, free access to the extracellular connexin loops which they mimic, would be expected to interact with these loops and perhaps obstruct the ATP-release conduit. Our work indeed confirmed this hypothesis and demonstrated a drastic inhibitory effect of gap 26 (mimicking a sequence on the first extracellular loop of the connexin-43 subunit; Table 9.1) on $InsP_3$-triggered ATP release.

Table 9.1 Peptides derived from connexin sequences

Peptide	Connexin	Residue Nos.	Sequence	Topographic position
Gap 26	43	63-75	VCYDKSFPISHVR	Extracellular loop I
Gap 27	43	204-214	SRPTEKTIFII	Extracellular loop II
Des 5	32	108-119	LEGHGDPLHLEEC	Intracellular loop II



An interesting finding is that gap 26 did not affect gap junctional coupling, at least when applied over a short period, e.g. 30 min (Fig. 9.2). This looks obvious given the fact that gap junction channels are composed of two hemichannels connected to each other by their extracellular loops, hence effectively shielding these loops for interactions with gap 26. Presumably, connexin mimetic peptides block gap junction channels by inhibiting the *de novo* formation of gap junctions rather then by blocking existing gap junctions. Consistent with this possibility is the fact that very long incubations with these peptides do indeed block gap junctional coupling (Krysko *et al.*).

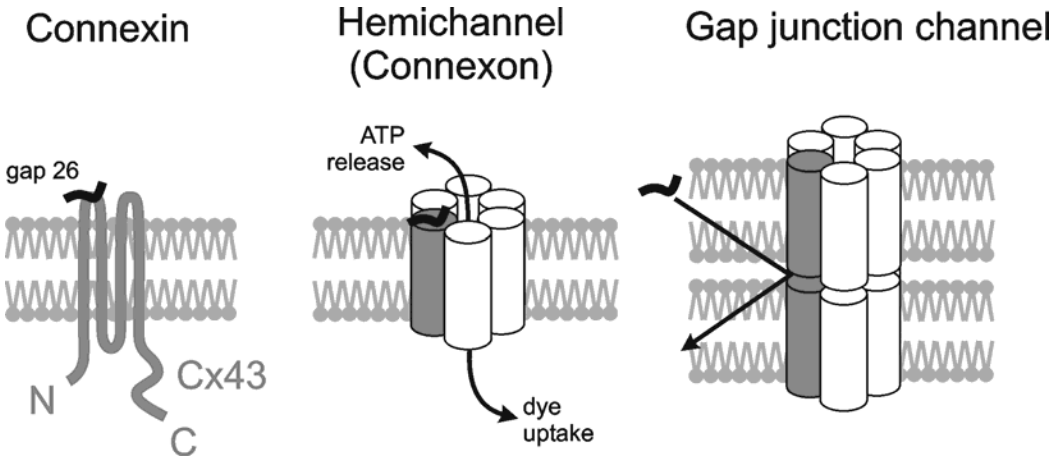


Fig. 9.2 Interactions of connexin mimetic peptides with connexin hemichannels and channels. The connexin mimetic peptides gap26 and gap 27 mimic a short (13 resp. 11 AA's) sequence on the first (gap 26) or second (gap 27) extracellular loop of connexin-43. These peptides are hypothesized to inhibit gap junctional coupling by preventing the assembly of two connexin hemichannels into a functional gap junction channel. Applied over a short period however, e.g. 30 min, we observed no inhibitory effects on coupling, yet very efficient inhibition of ATP release from the cells and dye uptake into the cells. This suggests that these peptides interact with the extracellular loops which are readily available in the hemichannel conformation to obstruct the channel or affect channel gating.

The most intruiging result of our work is the fact that $InsP_3$–triggered and connexin-related ATP release is calcium-dependent, suggesting the opening of connexin hemichannels in response to increased cytoplasmic calcium. Calcium-dependent opening of connexin hemichannels is however a controversial issue because increased calcium is since long known to close gap junction channels (Bruzzone *et al.*, 1994; Lazrak *et al.*, 1993; Spray *et al.*, 1992) (although there are exceptions; see Delage *et al.*, 1998). Clearly, this point needs further investigations in order to clarify the relation between calcium and connexin channel gating.

What are the mechanisms of cellular ATP release?

Our previous data indicate that an elevation of intracellular $InsP_3$ triggers ATP release that is connexin related. Evidence from electrophysiological work in isolated cells indicates that connexins can, in addition to their well known presence in gap junction channels, also appear as hemichannels (DeVries *et al.*, 1992). Further work with various types of stimuli, such as mechanical stimulation or exposure of the cells to low extracellular calcium solutions has demonstrated that these hemichannels can acts as a conduit to release ATP (Stout *et al.*, 2002) or to allow the entry of reporter dyes into the cells (Arcuino *et al.*, 2002). In chapter 6 we have applied various suggested blockers of connexin hemichannels and other putative ATP release pathways to further document the nature of the release process being activated by stimulation with $InsP_3$ and its subsequent calcium increase. The experiments showed a distinct picture of pharmacological sensitivity to the various blockers tested, with connexin mimetic peptides (gap 26 and gap 27; see table 9.1) and trivalent ions (lanthanum and gadolinium) inhibiting triggered ATP release without affecting gap junctional coupling and with gap junction blockers like α-GA blocking both coupling and triggered ATP release (summarized in Fig. 6.6). The pharmacological sensitivity of $InsP_3$-triggered ATP release was very similar to the sensitivity displayed by zero extracellular calcium-triggered ATP release, indicating that both triggers activate a similar release pathway. Only fenamates and NPPB had disparate effects on the two forms of triggered ATP release. In addition, zero calcium-triggered uptake of reporter dyes into the cells showed a pharmacological sensitivity that was very similar to the sensitivity of ATP release triggered by the same stimulus, indicating that the release pathway is permeable in both directions and thus diffusive in nature. Work with connexin-free and connexin transfected HeLa cell lines convincingly demonstrated that gap 27 only blocked triggered ATP release when connexin-43 was present. This indicates that the blocking effect of gap 27 on ATP release depends on the specific interaction of the peptide with the connexin-43 subunit. Finally, the disparate effects observed with NPPB or the fenamates indicate that there must be a difference between the two pathways of triggered ATP release. This difference might reside in the intracellular pathways interposed between zero calcium exposure

and the triggering of ATP release. The intermediary pathway involved is largely unknown, and thus further work will be needed to clarify the steps involved. Our preliminary work suggests that zero calcium exposure also incites the $InsP_3$ and intracellular calcium pathway.

What is the influence of TNF-α on the communication of calcium signals between blood-brain barrier cells?

In chapter 7 we investigated the influence of the pro-inflammatory cytokine TNF-α on the two pathways of calcium signal communication. The work showed that the cytokine inhibits these two pathways by inhibiting connexin-related communication pathways like gap junctions and connexin hemichannels. TNF-α induced basal release was not connexin-related based on its insensitivity to the connexin mimetic peptide gap 26. These results are compatible with our previous findings, in that a substance that inhibits gap junctional coupling also inhibits the triggered ATP release, adding further evidence to the involvement of connexin hemichannels in ATP release. A similar logic applies to the work with PKC stimulation: activation of the PKC pathway is known to inhibit gap junctions (Lampe *et al.*, 2000) and in our experiments it also inhibited the triggered ATP release. PKC thus mimicked the TNF-α effects and this furthermore suggests that the TNF-α effects are mediated by this kinase.

A potentially important aspect of our findings with TNF-α is that this substance blocked the first step in the paracrine signaling cascade, i.e. the release of ATP by the cells. Consequently, the cytokine will be expected to silence all forms of purinergic cell-to-cell signaling, including also non-calcium related cell-to-cell signaling. Given the ubiquity of purinergic receptor distribution, this might have important effects on signaling between all cell types present at the blood-brain barrier, including endothelial cells, smooth muscle cells, pericytes, astrocytes and blood cells. Further work will be needed to determine the importance of these suggested TNF-α effects on the communication and interactions of these cell types with the blood-brain barrier.

What intracellular $InsP_3$ concentrations and extracellular ATP amounts are involved in our experimental work?

Based on the notion that our standard photoactivation protocol to release $InsP_3$ in the cells needs rather high, i.e. micromolar concentrations of this messenger in order to trigger calcium waves and cellular ATP release, we aimed in chapter 8 to determine a concentration-response relation for

$InsP_3$-triggered ATP release. We approached this problem by a careful quantitative consideration of all technical steps involved in the loading and uncaging steps of the protocol. Our results indicate that the ATP response to $InsP_3$ elevation is not saturating in the order of 10 µM $InsP_3$ concentrations, suggesting that low affinity $InsP_3$ receptors are involved in activating ATP release. We also found that above ten micromolar $InsP_3$ stimulation triggered significant amounts of ATP estimated to be in the order of 1-2 % of the total cellular ATP content. This would mean that repeated application of this stimulus would be expected to significantly reduce the cellular ATP content, a possibility that should be tested in future work.

9.2. Perspectives for future experimental work

$InsP_3$ and its downstream calcium signal evoke intercellular calcium waves through ATP release while the calcium signal alone appears to be insufficient.

Intercellular calcium waves on the one hand and ATP release on the other hand are both dependent on intracellular calcium whereas an increase of calcium, to the same level as the calcium concentration reached with the $InsP_3$ trigger, appears insufficient. Further research is designed to elucidate the differences between both triggers and to determine the threshold calcium concentration required to evoke ATP release. Experiments will be performed to investigate the concentration-response relation between the photolytically triggered calcium increase and the ATP response. A membrane-anchored calcium probe will be used to monitor calcium concentration changes near the plasma membrane where the release sites are supposed to be localized.

How do $InsP_3$ and zero calcium trigger the release of ATP?

The differential effect of the fenamates on InsP3- versus zero calcium-triggered ATP release, points towards differences between these two different stimuli of triggered ATP release. Preliminary results indicate that zero calcium-triggered ATP release is dependent on an intracellular store-borne calcium increase. This suggests that both triggers stimulate ATP release in a calcium-dependent manner. Further work will be done to identify the intracellular calcium signaling pathways that are the most effective in triggering cellular ATP release. Inhibitor substances of InsP3- and ryanodine-sensitive stores will be applied to investigate their effect on zero calcium-triggered ATP release. In addition, direct stimulation of ryanodine receptors with cyclic ADP ribose will be applied to probe whether this is also an effective trigger of ATP release. We will furthermore investigate the possibility of involvement of other ATP release pathways such as vesicular release. We hypothesize that ATP release is mediated by the concerted actions of many different mechanisms linked to each other by a complex network of intermediate signals. The opening of connexin hemichannels might, for example, allow massive calcium influx thereby activating and mobilizing other pathways of ATP release.

Do these intercellular calcium signals contribute to the neurobarrier coupling?

Last but not least, we will try to validate the neurobarrier hypothesis and investigate the specific role of calcium signals in transfering neuronal information to the blood-brain barrier. Neuronal activity has been demonstrated to increase blood brain barrier glucose transport (Cornford *et al.*,

2000) while astrocytes sense increased neuronal activity by responding with calcium signals. Neuron-astrocyte-endothelial calcium signals might be instrumental in this pathway, at least if these signals are proven to influence the transport of glucose over the blood-brain barrier. We will design a new experimental setup to investigate whether photolytic manipulations acting at intracellular calcium have an influence on the transport of substances like glucose over an *in vitro* blood-brain barrier.

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