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Calcium Signaling in Development and Disease

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“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”

J.R.R. Tolkien

Till Sis…

Abstract

The calcium ion (Ca^{2+}) is a highly versatile signaling messenger involved in a diverse range of physiological processes such as gene transcription/expression, proliferation, differentiation and cell death. Intracellular Ca^{2+} signals are generated through a 10 000 – 20 000 fold gradient across the cell membrane and via release from the external milieu and/or internal Ca^{2+} stores. Cells have a unique signaling toolkit to control Ca^{2+} homeostasis including a selection of ion channels, pumps, exchangers and Ca^{2+} binding proteins.

We have reported that ouabain, an endogenous steroid hormone and ligand to the Na^{+},K^{+}-ATPase, can trigger dendritic growth in cortical neurons through signal transduction. This involves a Ca^{2+}-dependent transcriptional program regulated by CREB and CRE-mediated gene activation, primarily regulated through Ca^{2+}/calmodulin-dependent protein kinases. The process also includes Ca^{2+} oscillations and phosphorylation of mitogen-activated protein kinases (ERK 1/2). These data suggest a novel role for Na^{+},K^{+}-ATPase and Ca^{2+} in dendritic growth during development.

Previous work has shown that treatment with protein kinase C (PKC) inhibitors results in a prolonged Ca^{2+} increase leading to calpain activation and release of apoptosis-inducing factor (AIF). We have demonstrated that hyperpolarization-activated cyclic nucleotide-gated (HCN) channel 2 is responsible for the Ca^{2+} influx. The influx is regulated via dephosphorylation of a residue in the intracellular C-terminal. This data shows a novel role for HCN channel 2 in cell death and a new possible drug target.

Bladder cancer is overall one of the ten most common cancers. We have shown that treatment with *Bacillus Calmette-Guerin* (BCG), currently the most effective intravesical agent against bladder cancer, induces an intracellular Ca^{2+} increase and reduces cell proliferation in urinary bladder cancer (T24) cells. Store depletion by SERCA inhibition blocked the BCG-triggered signal, thereby suggesting a role of the endoplasmic reticulum as a Ca^{2+} source. This signaling event was dependent on phospholipas C since pharmacological inhibition or small interference RNA-mediated gene silencing abolished the response. Finally EdU incorporation revealed that BCG-controlled cell proliferation was mediated via a Ca^{2+}- and PLC-dependent signaling cascade.

In summary this thesis presents three studies highlighting three different roles for Ca^{2+} signaling. They show that Ca^{2+} signaling is involved in processes critical for cell differentiation, cell proliferation, and cell death, three aspects highly coordinated with development and disease.

List of Publications

I. **Desfrere L, Karlsson M, Hiyoshi H, Malmersjö S, Nanou E, Estrada M, Miyakawa A, Lagercrantz H, El Manira A, Lal M, Uhlén P (2009).** Na,K-ATPase signal transduction triggers CREB activation and dendritic growth. *Proc Natl Acad Sci U S A*;106(7), 2212-7

II. **Norberg E, Karlsson M, Korenovska O, Szydlowski S, Silberberg G, Uhlén P, Orrenius S, Zhivotovsky B (2010).** Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis. *EMBO J.*;29(22), 3869-78

III. **Karlsson M, Ibarra C, Kjällquist U, Zajac P, Lundgren K, Kaba R, Bavand-Chobot N, Linnarsson S, Wiklund P, Miyakawa A, and Uhlén P.**
Bacillus Calmette-Guerin (BCG) Triggers PLC-Dependent Ca^{2+} Signaling in Bladder Cancer Cells.
Manuscript

Contents

List of abbreviations

AIF	Apoptosis-inducing factor
ATP	Adenosine triphospate
BCG	*Bacillus Calmette*-Guerin
Ca^{2+}	Calcium
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CaSR	Ca^{2+}-sensing receptor
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
cGMP	Cyclic guanosine monophosphate
CICR	Ca^{2+} induced Ca^{2+} release
CNBD	Cyclic nucleotide binding domain
CREB	cAMP response element binding
CREST	Ca^{2+}-responsive transactivator
Cs^{+}	Cesium
CTS	Cardiotonic steroids
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinases
GPCRs	G-protein coupled receptors
HCN channel	Hyperpolarization-activated cyclic nucleotide-gated channel
ICAM	Intercellular adhesion molecules
IL	Interleukin
INF	Interferon
$InsP_3$	Inositol 1,4,5-trisphosphate
$InsP_3R$	Inositol 1,4,5-trisphosphate receptor
K^{+}	Potassium
MAP kinase	Mitogen activated protein kinase
MLS	Mitochondrial localization signal
Na^{+}	Sodium
NCX	Na^{+}/Ca^{2+} exchanger
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-B
NK celler	Natural killer cells
NMDA	N-methyl d-aspartate
PGN	Peptidoglycan
PIP	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTX	Pertussis toxin
PMCA	Plasma membrane Ca^{2+}-ATPase
ROCCs	Receptor operated Ca^{2+} channels

RyR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SMAC	Second mitochondrial activator of caspases
SMOCs	Second messenger operated Ca^{2+} channels
SR	Sarcoplasmic reticulum
STS	Staurosporine
TCR	T-cell receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRP channel	Transient receptor potential channel
VOCCs	Voltage-dependent Ca^{2+} channels

1 Introduction

1.1. Calcium signaling

”Kalzium? Ja, das ist alles!”
O. Loewi

The foundation for our current understanding of the multiple roles of calcium ions (Ca^{2+}) was established by Sydney Ringer in the late 19th century. Ringer discovered that Ca^{2+} ions are crucial for regulation of heart contraction through the finding that a frog heart was able to beat when put in tap water but not in distilled water. Around the same period of time he also found that Ca^{2+} ions are involved in other processes like regulation of fertilization, tadpole development and to determine survival of fishes (Ringer, 1883a; Ringer, 1883b; Ringer, 1886; Ringer, 1890; Ringer and Sainsbury, 1894). Today the understanding and knowledge of Ca^{2+} signaling and its role in cellular processes is considerably broader.

Ca^{2+} is one of, if not the most versatile second messenger signaling molecule. It is involved in the activation/regulation of a wide range of physiological processes such as differentiation, exocytosis, gene expression/transcription, memory, muscle contraction, proliferation and cell death (Berridge et al., 2003). The great diversity of Ca^{2+} signals associated with these processes is a result of complex regulation mechanisms of Ca^{2+} signals (i.e. Ca^{2+} concentration within the cell and changes in the concentration) in space, time and amplitude (Berridge et al., 1998; Boulware and Marchant, 2008).

1.1.1. Calcium signaling toolkit

A healthy cell at rest normally exhibits a cytoplasmic Ca^{2+} concentration around 100 nM while the extracellular concentration is between 1 and 2 mM, which creates a 10 000 – 20 000 fold Ca^{2+} gradient across the membrane. Upon activation the cytoplasmic concentration can rise to about 1000 nM. Different cytoplasmic compartments have a unique Ca^{2+} concentration (Figure 1.).

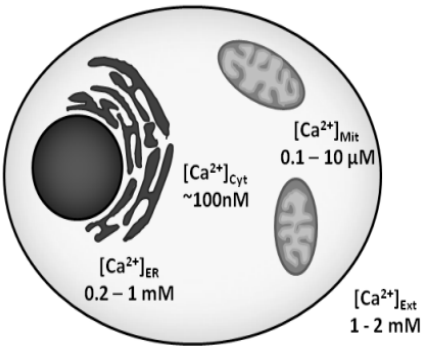

Figure 1. Cellular Ca^{2+} homeostasis

The Ca^{2+} homeostasis within a cell is a result of highly coordinated signaling via a large number of Ca^{2+} regulators (Box 1.). Each cell type expresses a unique set of regulators. This set of regulators is not totally fixed and can be remodeled depending on the need. Furthermore the signaling toolkit can more or less be divided into four different parts:

1. **Encoding** – The conversion from stimuli to a Ca^{2+} signal
2. **The ON mechanisms** – the Ca^{2+} influx resulting in an elevation of intracellular Ca^{2+}
3. **Decoding** – the process which transforms the encoded information from the Ca^{2+} signal into the physiological target response
4. **The OFF mechanism** – the removal of Ca^{2+} to restore basal intracellular Ca^{2+} levels

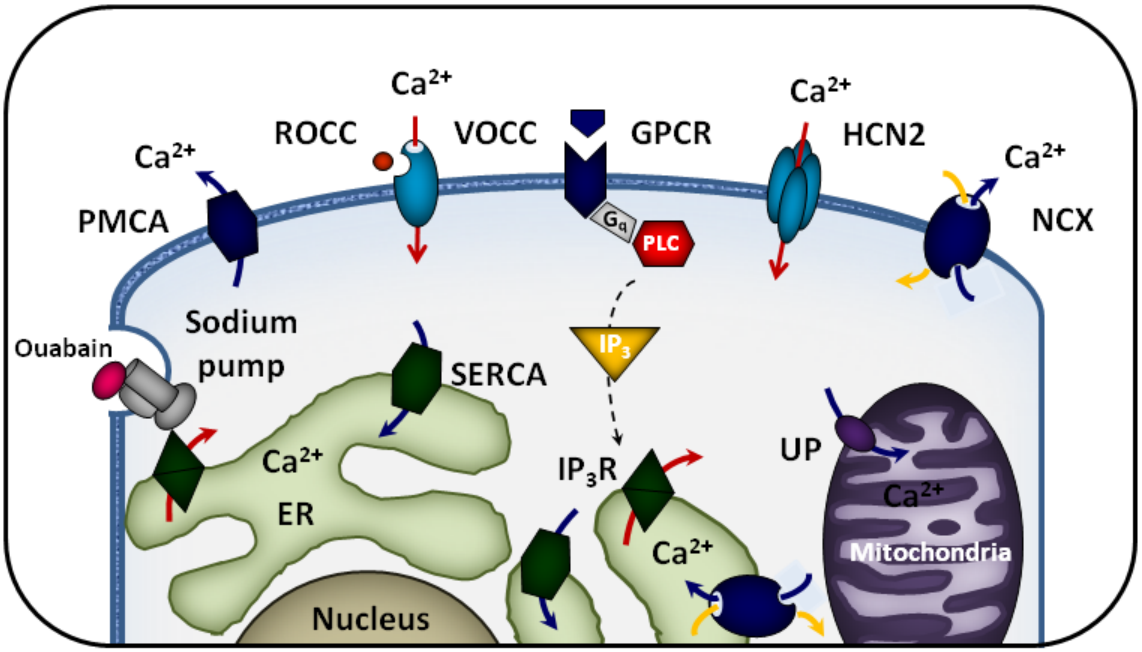

Figure 2. Cellular regulators for Ca^{2+} signaling

1.1.1.1. Encoding

The encoding process involve the generation of Ca^{2+} signals in response to a diverse range of stimulus. Here it refers to activation of channels permeable to Ca^{2+} e.g. Inositol 1,4, 5-trisphosphate ($InsP_3$) will activate Inositol 1,4,5-trisphosphate receptor ($InsP_3R$) that in turn will initiate the ON mechanism.

1.1.1.2. The ON mechanism

An elevation of intracellular Ca^{2+}, also referred to as the "ON" mechanism, can be generated from two sources; the external milieu and/or internal stores. Ca^{2+} can enter the cell from outside via a variety of plasma membrane channels. The most studied type of channel is voltage-operated Ca^{2+} channels (VOCCs). They are found in excitable cells and generate a fast Ca^{2+} flux due to activation through depolarization of the plasma membrane, which occurs for example during muscle contraction and exocytosis. Another group of channels are the receptor-operated Ca^{2+} channels (ROCCs) which are activated via binding of external stimuli to the receptor. An example is the NMDA receptor that is activated by glutamate in neurons. A third

type of channels is the second messenger-operated Ca^{2+} channels (SMOCs). These are regulated by intracellular messengers (cAMP, cGMP) and include the cyclic-nucleotide gated channels found in sensory systems and hyperpolarization-activated cyclic nucleotide-gated channels found in neurons and cardiomyocytes. Beyond the range of channels mentioned here, there are other channels also contributing to the Ca^{2+} homeostasis in the cell (Box 1) (Berridge et al., 2003; Bootman et al., 2001).

The release of Ca^{2+} from internal stores mainly occurs from the sarco-/endoplasmic reticulum (SR/ER) through $InsP_3R$ and ryanodine receptor (RyR). They can be regulated by a variety of factors but are also sensitive to Ca^{2+} itself and are therefore involved in Ca^{2+} induced Ca^{2+} release (CICR) (Berridge et al., 2003; Bootman et al., 2001; Mikoshiba, 2007).

1.1.1.3. Decoding

After the ON mechanism has generated a Ca^{2+} signal, the signal is decoded in order to activate a physiological response. Ca^{2+} is an ion and cannot itself be modulated. Instead the binding of Ca^{2+} to Ca^{2+}-binding proteins can modulate the conformation and charge of the protein and thereby alter its function. Ca^{2+}-binding proteins can be divided into two groups; Ca^{2+}-sensors and Ca^{2+}-buffers. Ca^{2+}-sensors will undergo conformational changes due to Ca^{2+} binding and are involved in the decoding process and activate different cellular processes. In contrast Ca^{2+} buffers will only undergo a minor conformational change and therefore function as buffers or transporters.

One of the most global sensor proteins is calmodulin. Calmodulin interacts with more than 100 different target proteins and is thus involved in the regulation of many different processes like gene transcription, ion channel modulation, muscle contraction, crosstalk among signaling pathways etc (Berridge et al., 2000).

Calmodulin has four EF hands that can bind Ca^{2+}, two in the N-terminal and two in the C-terminal. Interestingly the C-terminal EF hand pair has a three- to five-fold higher affinity for Ca^{2+} than for the N-terminal pair. Calmodulin partially performs it tasks by regulating diverse effector proteins. Binding to effector proteins will increase calmodulins affinity for Ca^{2+} and thereby change the Ca^{2+} content in the complex. Protein kinases, phosphodiesterase, calcineurin, RyR, $InsP_3R$ are examples of effector proteins regulated by calmodulin and they can be divided into different classes depending on their mode of regulation in the absence or presence of Ca^{2+}.

Calmodulin can be regulated by Ca^{2+} in three different levels; cellular, intermolecular and sub-molecular. First, at cellular level changes in intracellular Ca^{2+} redistribute calmodulin within the cell. In muscle cells and neurons an influx of Ca^{2+} can lead to

intracellular redistribution from the cytoplasm to the nucleus. Second, at intermolecular level it will be regulated by supporting different types of interactions with a variety of effectors e.g. different downstream proteins. Third, when Ca^{2+} binds to calmodulin the conformation is changed which triggers target-specific activation. An example of this is calcineurin which forms an inactive low-affinity complex with calmodulin during low Ca^{2+} concentrations. In contrast in high Ca^{2+} concentrations the complex will adopt a high-affinity complex and calcineurin and will be activated by calmodulin (Chin and Means, 2000).

Other Ca^{2+}-sensor proteins can have more specific functions, for example synaptotagmin which regulates exocytosis (Zhang et al., 2010).

1.1.1.4. The OFF mechanism

When the desired signal is achieved the basal Ca^{2+} levels are restored through the "OFF" mechanism. This includes the plasma membrane Ca^{2+}-ATPase (PMCA), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), Na^{+}/Ca^{2+} exchanger (NCX), mitochondrial uniporter and binding to cytosolic buffering proteins (Berridge et al., 2003; Berridge et al., 2000; Guerini et al., 2005; Wuytack et al., 2002). The off mechanism is crucial since elevated Ca^{2+} levels for longer times is toxic for the cell and can induce cell death.

The PMCA pump is a transmembrane protein found in the plasma membrane that belongs to the family of P-type ATPases and exists in two conformational states (E1 and E2). The E1 state, where the Ca^{2+} binding site is directed toward the cytoplasm and binds Ca^{2+} with high affinity and the E2 state where Ca^{2+} is released to the external milieu by decreased Ca^{2+} affinity. Four PMCA (PMCA1-4) isoforms have been discovered; PMCA1 and PMCA4 are ubiquitously expressed whereas PMCA2 and PMCA3 are primarily expressed in the nervous system (Guerini et al., 2005).

The NCX has nine transmembrane segments and is found in the plasma membrane and in the outer mitochondrial membrane. The task for the NCX is to export one Ca^{2+} in the exchange of 3 Na^{+}, using the Na^{+} gradient as an energy source. On the other hand the direction of the NCX transport can be reversed if necessary e.g. if the Na^{+} gradient decreases and/or the membrane potential shifts towards a more positive value. The NCX exist in three isoforms, NCX1-NCX3. NCX1 is expressed in the heart, NCX2 in the brain and NCX in skeletal muscles (Guerini et al., 2005; Kimura et al., 2009; Quednau et al., 1997).

The SERCA pump is also a transmembrane protein belonging to the P-type ATPase although it is found in the membrane of sarco/endoplasmic reticulum where it pumps Ca^{2+} from the cytoplasm in to the SR/ER. The pump mechanism includes

transport of two Ca^{2+} ions across the membrane utilizing the energy from one ATP hydrolysis. Mammals express three SERCA genes which are spliced into a range of isoforms. Today at least 10 different isoforms has been detected at the protein level. SERCA1 and its two isoforms are mostly expressed in fast skeletal muscle, SERCA 2 including its three isoforms are mainly found in cardiac tissue and slow skeletal muscles while SERCA3 and its six isoforms are expressed in several non-muscle tissues and have been found in hematopoietic cell lineages, platelets, epithelial cells, fibroblasts, and endothelial cells (Periasamy and Kalyanasundaram, 2007; Vangheluwe et al., 2005; Wuytack et al., 2002).

The mitochondrial uniporter is found in the inner mitochondrial membrane. It is slower than other ion channels and has a turnover number 1000-fold lower than an ion channels ($>10^5$ s^{-1}). The Ca^{2+} transport is in line with the electrochemical gradient sustained across the inner membrane and does not involve ATP utilization or co-transport of other ions (Kirichok et al., 2004).

Both the PMCA and SERCA pumps have low transport rates although Ca^{2+} binds with high affinity and thus they are able to respond to small changes in Ca^{2+} concentration. In contrast the NCX and mitochondrial uniporter have high transport rates and lower affinity and consequently limits Ca^{2+} transient over a wider dynamic range.

The Ca^{2+} buffering proteins that become loaded during the ON mechanism become unloaded during the OFF mechanism. However, cytosolic buffers can modulate both the amplitude and duration of Ca^{2+} signals. Three buffer proteins are calretiinin, calbindin D and parvalbumin. The two first are fast buffers while parvalbumin is acting slower but binds Ca^{2+} with higher affinity (Berridge et al., 2003; Berridge et al., 2000; Carafoli et al., 2001; Ikura et al., 2002; Lewit-Bentley and Rety, 2000).

Box 1. Ca^{2+} signaling regulators

ON MECHANISM	
Channels	
Voltage-operated channels (VOCs)	L-, P/Q-, N-, R- and T-type
Receptor-operated channels (ROCs)	NMDA receptors, ATP receptors
Second-messenger-operated channels (SMOCs)	CNG, HCN
Transient receptor potential (TRP) ion-channels	TRPC1-7, TRPV1-6,TRPM1-8
Inositol-1,4,5-trisphophate receptors ($InsP_3Rs$)	$InsP_3R1$-3
Ryanodine receptors (RyRs)	RyR1-3

OFF MECHANISM	
Pumps and exchangers	
Plasma membrane Ca^{2+}-ATPases (PMCAs)	PMCA1-4
Sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs)	SERCA1-3
Na^+/Ca^{2+} exchangers (NCXs)	NCX1-3
Mitochondrial channels and exchangers	Uniporter, NCX
Golgi pumps	SPCA1, SPCA2

OTHER MEDIATORS	
Receptors	**GPCR:** muscarin-, antgiotensin-, bradykinin-, histamine-, metabotropic glutamate-, extracellular Ca^{2+} sensing-, leukotrien-, neurotensin receptors **Tyrosine-kinase-linked:** platelet-derived growth factor receptors, epidermal growth factor receptors
Transducers	G-proteins, phospholipase C (PLC), ADP ribosyl cyclase, Regulators of G-protein signaling
Calcium buffers	**Cytosolic:** CalbindinD-28, celretinin, parvalbumin, **ER/SR:** calnexin, calreticulin, GRP 78
Calcium effectors	Calmodulin, troponin C, synaptotagmin, S100, annexin, meuronal Ca^{2+} sensor, hippocalcin
Calcium-sensitive enzymes and processes	**Ca2+ regulated enzymes :** CaMK, myosin ligh chain kinase, phosphorylase,Adenylyl cyclase, PYK2, PKC,nitric oxide synthase, calcineurin, phophodiesterase, **Transcription factors:** NFAT, CREB, CBP, DREAM

1.1.2. Temporal and spatial input on calcium signaling

Different spatial and temporal aspects contribute to the versatility of the Ca^{2+} signaling. Intracellular Ca^{2+} signals can occur in many shapes and forms but can very generally be grouped into three classes; transients, oscillations and sustained signals (Uhlen and Fritz, 2010). Ca^{2+} oscillations refers to multiple repeats of Ca^{2+} peaks that

can have different modulation of frequency and amplitude and can be induced by compounds like α-haemolysine (Uhlen et al., 2000), ouabain (Aizman et al., 2001) and testosterone (Estrada et al., 2006).

Timing is central for Ca^{2+} signaling. Distinct processes can have a remarkable variety in the temporal range, lasting microseconds (e.g. exocytosis), minutes or hours (e.g. gene transcription, differentiation, proliferation) or even up to months or years (e.g. memory processes). Depending on the temporal pattern different types of cytoplasmic Ca^{2+} signal will arise. However, this is dependent on how the encoding process interpreted the signals and transferred them into cytoplasmic Ca^{2+} signals. Furthermore the frequency of the signal contributes towards the selection of which effectors that will be activated and thereby vary the physiological output of the signal (Boulware and Marchant, 2008). Two proteins that are involved in decoding the frequency are CaMKII and PKC (De Koninck and Schulman, 1998; Oancea and Meyer, 1998). Dolmetch *et al*. demonstrated that low frequency Ca^{2+} signals activates NF-κB while NFAT was activated at a higher frequency rate (Dolmetsch et al., 1998).

Ca^{2+} signals can have spatial ranges from nanometer to centimeter. Ca^{2+} "blips", "puffs" and waves (more discussed in next section) are example of Ca^{2+} signals with different spatial pattern (Bootman et al., 1997).

The amplitude of Ca^{2+} signals can also be interpreted into information. However because of the difficulty to distinguish small Ca^{2+} peaks from the background noise, signals that are modulated through changes in the amplitude are less reliable (Berridge et al., 1998).

1.1.3. Inositol 1,4,5-trisphosphate receptor and Inositol 1,4,5-trisphosphate

In 1979 a protein named P400 was purified by Mikoshiba *et al*. which was later identified as the Inositol 1,4,5-trisphosphate receptor ($InsP_3R$) (Maeda et al., 1988; Mikoshiba et al., 1979). Its main regulator Inositol 1,4,5-trisphosphate ($InsP_3$) was discovered in the early 1980s (Streb et al., 1983).

The $InsP_3R$ is an intracellular ion channel found in the SR/ER membrane. It is a complex formed of four subunits (313 kDa each) that each consists of six membrane-spanning helices that contribute to the ion-conducting pore. The cytosolic NH_2 part can be divided into an $InsP_3$ binding domain and a regulatory/suppressor domain. The suppressor domain will interact with the $InsP_3$ binding domain and suppress the binding affinity for $InsP_3$ (Mikoshiba, 2007).

Three different isoforms of $InsP_3R$ have been identified in mammals, $InsP_3R1$-$InsP_3R3$. Multiple isoforms are found in most cell types (Blondel et al., 1994; Yamada et al., 1994; Yamamoto-Hino et al., 1994). Each isoform has a unique affinity for InsP3 (Newton et al., 1994). The binding of the suppressor domain to the binding domain is responsible for the isoform specific affinity for $InsP_3$ (Mikoshiba, 2007).

As mentioned earlier, $InsP_3R$ is involved in the Ca^{2+}influx during the ON mechanism. Stimulation of cell-surface receptors (for example GPCRs or TKRs) activates phospholipase C (PLC). Active PLC in turn hydrolyses phosphatidylinositol bisphosphate (PIP_2) to $InsP_3$ and diacylglycerol (DAG). $InsP_3$ will diffuse in the cell and bind to $InsP_3R$ in the SR/ER and results in an intracellular Ca^{2+} rise. Depending on the status of the receptor, different types of Ca^{2+} responses are generated (Fill, 2003; Foskett et al., 2007; Mikoshiba, 2007).

Three different types of Ca^{2+} responses generated by $InsP_3R$ are blips, puffs and waves. A Ca^{2+} blip (last around 200 ms, has an amplitude < 30 nm and diffuse less than 2 micrometers) is generated from opening of a single $InsP_3R$. Another response generated from clusters of 40-70 $InsP_3R$ is a Ca^{2+} puff (last for 500 ms, has an amplitude around 200 nm and is spread over 6 µm) (Bootman et al., 1997; Thomas et al., 1998). Finally a Ca^{2+} wave can be produced. This wave is produced through Ca^{2+} puffs that are spread within the cell (Mikoshiba, 2007).

In addition to $InsP_3$, $InsP_3R$ is regulated by Ca^{2+}. The $InsP_3$ released Ca^{2+} can in turn further promote activation of more $InsP_3R$ channels that release additional Ca^{2+} as a positive feedback. This phenomenon is called Ca^{2+} induced Ca^{2+} release (CICR) and is used to coordinate communication and activities among channels. CICR is involved in the formation of puffs and waves (Foskett et al., 2007). In addition to $InsP_3R$ the RyR is also able to accomplish CICR.

Conversely, in addition to stimulating the $InsP_3R$ channel, Ca^{2+} can also have an inhibitory effect. The $InsP_3R$ cannot release Ca^{2+} if the intracellular concentration is to low (< 50 nM) or to high (10-100 µM) (Berridge et al., 2000; Foskett et al., 2007). However, evidence suggests that high Ca^{2+} concentration does not always have inhibitory effects and that the relationship may be more sigmoid than bell-shaped (Bootman and Lipp, 1999).

1.1.4. Implication of calcium signaling in life and death

Ca^{2+} signaling is involved in different cellular processes throughout life from fertilization until death. Some of these processes will be mentioned here. However the implications of Ca^{2+} signaling in neuronal development, cell death and cancer will be discussed further in separate sections.

1.1.4.1. Fertilization

Ca^{2+} is involved in mammalian fertilization via the $InsP_3/Ca^{2+}$ pathway. Ca^{2+} oscillations are triggered when the sperm interacts with the egg. The sperm fusion with the oocyte activates PLCγ, which will hydrolyze PIP_2 to $InsP_3$ and DAG. $InsP_3$ in turn will stimulate $InsP_3R$ and Ca^{2+} release from internal stores and CICR will sustain the oscillations for a couple of hours (Miyazaki et al., 1993).

1.1.4.2. Differentiation

In the early embryo development Ca^{2+} signaling initially contributes to the body polarity and pattern formation. Later in development, Ca^{2+} is involved in and controls the differentiation of specific cell types. Differentiation of cells into a special cell type requires various signals. Therefore it is important to generate a toolkit for each cells requirement. Ca^{2+} oscillation are for example involved in the differentiation of muscles and neurons (Buonanno and Fields, 1999). NFAT, a Ca^{2+} dependent transcription factor, is involved in the formation of septum and valves in the heart (de la Pompa et al., 1998).

1.1.4.3. Proliferation

Ca^{2+} is also involved in proliferation and in the regulation of the cell cycle, mainly in G1 and at the G1/S and G2/M transitions, via upstream regulation and activation of transcriptions factors involved in cell cycle (Roderick and Cook, 2008). The role of Ca^{2+} during proliferation is well studied in lymphocyte activation. Antigen binding to surface receptors (TCR) of the lymphocytes activates PLCγ and $InsP_3$ is produced. $InsP_3$ will recruit Ca^{2+} via $InsP_3R$ from internal stores and when the stores are empty there will be an external influx of Ca^{2+} via store operated Ca^{2+} channels (Feske, 2007).

1.1.4.4. Activation of transcription factors

As mentioned above Ca^{2+} often controls the cellular fate via regulation and activation of transcription factors. Transcription factors that are regulated by Ca^{2+} during the cell cycle include FOS, JUN, MYC, NFAT (Roderick and Cook, 2008). In lymphocytes, Ca^{2+} stimulates calcineurin to dephoshorylate NFAT. Dephosphorylation of NFAT will translocate it to the nucleus and transcription of different genes can occur. CREB is another transcription factor that is regulated by Ca^{2+} (Berridge et al., 2000).

1.2. Calcium and neural development

1.2.1. Neural development

The neural development involves a huge amount of cellular and molecular signaling processes that will generate, shape and reshape the nervous system from early stages of embryogenesis until the end of life. The growth, branching and guidance of neural projections during development are controlled by complex mechanisms with both diffusible and local acting signaling molecules binding specific receptors. The neural projections will form axons or dendrites that are specialized for communication and will form synaptic contacts with other nerve cells to allow nerve impulses to be transmitted.

1.2.2. The role of calcium in dendritic growth

The involvement of Ca^{2+} in dendritic growth is comparatively a quite recent finding. Spontaneous Ca^{2+} activity occurs during a period of intense dendritic growth in neurons and emerging evidence suggests that Ca^{2+} is needed for the dendritic growth and branching throughout this period.

An increase in intracellular Ca^{2+} is necessary to gain stability in the branching of embryonic retinal ganglion cells. First synaptic transmission contributes to an elevated intracellular Ca^{2+} level. This elevation, originating from the extracellular milieu, will induce a Ca^{2+}-induced Ca^{2+} release (CICR) from internal stores which will contribute to stabilization of the branches (Lohmann et al., 2002).

In addition, an intracellular Ca^{2+} elevation can affect dendritic growth via downstream regulators including for example Ca^{2+}/calmodulin-dependent protein kinase (CaMK). CaMKII is a neuronal signaling protein highly expressed in the brain. CaMKIIβ is required to initiate branching of dendrites in both sympathetic neurons (Vaillant et al., 2002) and hippocampal neurons (Fink et al., 2003). However CaMKIIα restricts dendritic growth in frog tectal neurons and mammalian cortical neurons (Redmond et al., 2002; Wu and Cline, 1998).

Another CaM kinase, CaMKIV which normally localized in the nucleus has also been reported to be involved in dendritic growth. Early cortical neurons require a Ca^{2+} influx through VOCCs to induce dendritic growth. However this growth was suppressed by a dominant negative form of CaMKIV and enhanced by a constitutively active form of CaMKIV. This effect was mediated through the transcription factor cAMP-response element binding protein (CREB) although activation of CREB alone is not sufficient to stimulate dendritic growth (Redmond et al., 2002).

In the search for other transcription factors involved in dendritic growth CREST (calcium-responsive transactivator) was found (Aizawa et al., 2004). CREST is a CREB-binding protein (CBP) highly expressed in the developing brain (Hardingham et al., 1999). Analysis of a CREST knockout mice brain showed defects in the dendritic growth in both cortex and hippocampus. In addition cortical neurons from CREST mutant animals displayed a reduced dendritic growth in response to depolarization stimuli. These data support a role for CREST in calcium-dependent dendritic growth (Aizawa et al., 2004).

In addition to different transcription factors, the mitogen-activated protein (MAP) kinase signaling pathway has been implicated in dendritic growth. Activation of this pathway, via sustained activation of ERK 1/2, is crucial for protrusion and stabilization of new dendritic filopodia in hippocampus (Wu et al., 2001). Further activation of ERK1/2 is implicated during dendritic growth in both sympathetic neurons (Vaillant et al., 2002) and in cerebellar granule cells (Borodinsky et al., 2003).

The extracellular calcium-sensing receptor (CaSR) monitors the extracellular Ca^{2+}level in several organs involved in Ca^{2+} homeostasis (e.g. parathyroid and thyroid glands). Furthermore, CaSR has been shown to be expressed in the developing nervous system although their functions there have been questioned. In 2008 Vizard et al. demonstrated that the CaSR has a crucial role in regulating the growth of neuronal processes in both the peripheral and central nervous systems. They found that CaSR are expressed in perinatal sympathetic neurons and the neurite growth was affected *in vitro* by altering the Ca^{2+} concentration via addition of CaSR agonists and antagonist. Similarly primary cultures from CaSR knockout mice revealed smaller neurite arbors compared to the wild-type control. *In vivo* data showed reduced density of sympathetic innervations of the iris CaSR knockout mice. Furthermore it was shown that hippocampal pyramidal neurons in mice, also expressing CaSR, had smaller dendrites after transfection with a dominant negative CaSR in organotypic cell cultures (Vizard et al., 2008). This study suggests that Ca^{2+} not only acts intracellularly but also extracellularly during neural development.

1.3. Calcium and cell death

1.3.1 Cell death

Cell death is a complex process with an extensive variety of mediators. It is a procedure that is essential for all living organism to be able to develop, grow and finally survive in an optimal way. There are several different types of cell death (some described below) which can occur alone or simultaneously. How the cell death occurs is determined by various factors for example different noxious signals, ATP concentration, cell type (Galluzzi et al., 2007).

Apoptosis or type 1 cell death is a highly regulated form of cellular self-destruction, having a critical role in embryonic development, normal tissue homeostasis in adults and in the immune system. Early morphological signs of apoptosis are chromatin condensation and cell shrinkage. Later in the process the nucleus and cytoplasm undergoes fragmentation with blebbing of the plasma membrane. In the end, the cell will break up and form apoptotic-bodies that are recognized and engulfed by phgocytic cells (Kerr et al., 1972). However, the fragmentation of the DNA might not always occur (Galluzzi et al., 2007). Another type of cell death is **autophagic cell death** (type 2). It is a rather slow process where the cytoplasm is confiscated inside double-membrane vacuoles which result in a massive vacuolization of the cytoplasm. The vacuoles are finally digested by lysosomal hydrolases (Kroemer and Jaattela, 2005). A third type of cell death, **necrosis** (type 3), is characterized by irreversible cytoplasm and organelle swelling and rupture of the plasma membrane. The release of the cellular content to the neighboring tissue can stimulate a local inflammation. Necrosis has for a long time been distinguished from apoptosis by being considered as an unregulated process. Although today evidence suggests that necrosis can also take place in a more controlled procedure called necroptosis (Vanlangenakker et al., 2008).

1.3.2. The role of calcium in cell death

As mentioned before Ca^{2+} signaling has an important role in regulation the cellular fate and including regulation of cell death. The same Ca^{2+} signaling system operating in a normal cell is involved in cell injury. The first report that Ca^{2+} was involved in cell death was by Fleckenstein et al in 1974. They suggested that the overload in intracellular Ca^{2+} in myocytes might be part of the mechanism that causes the cardiac pathology seen after ischemia (Fleckenstein et al). Next, Nicholson *et al*. demonstrated that the substantial Ca^{2+} influx from the extracellular space into neurons had a role in the ischemic cerebellum (Nicholson et al., 1977). During the last 30 years the role of Ca^{2+} in different cell death pathways has been further established (reviewed by: (Choi, 1995; Eisner et al., 2006; Ermak and Davies, 2002;

Kristian and Siesjo, 1998; Nicotera and Orrenius, 1992; Norberg et al., 2008; Orrenius and Nicotera, 1994)).
Following a cell injury pathological Ca^{2+} signals are generated. Usually these signals appear as a prolonged intracellular Ca^{2+} elevation. The Ca^{2+} elevation can be a result from Ca^{2+} overstimulation and/or failure to maintain Ca^{2+} homeostasis through an increased Ca^{2+} entry/release, compromised Ca^{2+} extrusion or a combination of these. The pathological Ca^{2+} signals can activate several intracellular pathways that trigger cell death and thus are involved in the selection of death process (apoptosis or necrosis). The choice of death process is decided by the severity of the injury and the status of the cell e.g. the resting concentration of ATP and the mitochondrial status (Orrenius et al., 2003).

The mitochondria are involved in the regulation of a diversity of processes like ATP synthesis, steroid hormone synthesis, metabolism and Ca^{2+} homeostasis. A Ca^{2+} increase in the cytoplasm will also induce a mitochondrial Ca^{2+} uptake, mostly driven by mitochondrial inner membrane potential. This will lead to elevated Ca^{2+} levels in the mitochondrial matrix although the level will quickly return to basal levels when the intracellular elevation decreases (Duchen, 1999). The mitochondrial Ca^{2+} concentration is determined by uptake through the uniporter, the efflux through NCX and Ca^{2+} buffering activity in the matrix. These three things make it possible for the mitochondria to tightly control the Ca^{2+} homeostasis and also be involved in the regulation of spatial and temporal pattern of Ca^{2+} signals (Szabadkai et al., 2006).

However mitochondrial Ca^{2+} can also be involved in cell death through permeability transition. A tolerant Ca^{2+} load in the mitochondrial matrix will result in opening of a permeability transition pore and thereby increase the permeability of the mitochondrial inner membrane to ions and solutes (≤ 1.5 kDa). This is most often followed by depolarization with Ca^{2+} release, matrix swelling and rupture of the outer mitochondrial membrane followed by release of cytochrome c and other apoptogenic proteins as AIF (Bernardi et al., 2006).

1.3.3. Pathological calcium signals

Extracellular influx of Ca^{2+} can quickly result in a toxic overload of Ca^{2+}. Spontaneous cells death occurs during mammalian spermatogenesis through a massive Ca^{2+} influx through VOCCs (Barone et al., 2004). VOCCs are also involved in chromaffin cell death where a Ca^{2+} elevation through L-type VOCCs lead to mitochondrial disruption and cell death (Cano-Abad et al., 2001). Another example of channels involved in cell death is ROCCs. The glutamate receptor NMDA can trigger pathological Ca^{2+} overload in neuronal excitotoxic cell death (Hardingham, 2009). Finally transient receptor potential (TRP) channels have been shown to be involved in cell death processes in photoreceptors (Yoon et al., 2000).

Internal Ca^{2+} stores mostly ER also plays a role in pathological Ca^{2+} signals and cell death. Failure in ER Ca^{2+} handling will affect chaperones, alter protein folding which will introduce an ER stress response that in severe cases can result in cell death (Pahl and Baeuerle, 1997). Disturbance in ER Ca^{2+} homeostasis or gathering of proteins within the ER will activate caspase 12 which will stimulate apoptosis (Nakagawa and Yuan, 2000). One other example is $InsP_3R$ which is involved is during neuronal excitotoxic cell death, caused by hyper-activation of the NMDA receptors, where $InsP_3R$ activation aggravated the cell death (Mattson et al., 1989). $InsP_3R$ mediated Ca^{2+} release can also induce increases in mitochondrial Ca^{2+} which in turn can lead to release of different pro-apoptotic factors like cytochrome c, SMAC (second mitochondrial activator of caspases) and apoptosis-inducing factor.

1.3.4. Apoptosis-inducing factor

Apoptosis-inducing factor (AIF) is a mitochondrial protein with a double nature in controlling both cellular life and death. AIF functions as an anti-apoptotic factor via its NADH oxidase activity in healthy cells (Klein et al., 2002), whereas it also can function as a pro-apoptotic factor by its unique ability to induce caspase-independent apoptosis (Joza et al., 2001).

AIF is transcribed in the cytoplasm forming a 67 kDa precursor protein containing a mitochondrial localization signal (MLS) in the N-terminal domain. When imported into the mitochondria, the protein is processed by a mitochondrial peptidase to a 62 kDa mature protein through removing the MLS (Otera et al., 2005). Within the mitochondria AIF is located in the intermembrane space where it is anchored in the N-terminal to the inner mitochondrial membrane (Otera et al., 2005). Removal of the MLS also exposes the flavine adenine nucleotide (FAD) domain which is required for its NADH oxidase activity (Miramar et al., 2001).

Upon stimulation with specific death signals, AIF can be further processed and cleaved from the inner mitochondrial membrane by proteases (calpains) generating a 57 kDa soluble protein. Permeabilization of the mitochondrial outer membrane admits release of AIF to the cytoplasm (Otera et al., 2005; Susin et al., 1999). This process requires an increase in intracellular Ca^{2+} to occur (Norberg et al., 2008).

Once released to the cytoplasm, AIF is translocated to the nucleus where it contributes to chromatin condensation and DNA fragmentation although the exact mechanism by which AIF acts remains unknown (Otera et al., 2005; Susin et al., 1999). However, the important role of AIF in cell death has only been shown in certain types of cells such as neurons and some tumor cells (Norberg et al., 2010b).

1.4. Calcium signaling and cancer

1.4.1. Cancer

Cancer (or malignant neoplasm) is a term used for a group of diseases that displays uncontrolled growth due to defect mechanisms in cell proliferation and cell death. The cell growth can be limited to a certain location (e.g. an organ or tissue) or be invasive into surrounding tissues and sometimes also metastasis to more distal parts. The emergence of a tumor is a multistage process and genetic abnormalities and environmental factors are the two contributing factors.

1.4.2. The role of calcium in cancer

As mentioned above a tumor is formed by excessive cell proliferation in combination with evading cell death resulting in an imbalance in the tissue homeostasis and thereby uncontrolled cell growth (Hanahan and Weinberg, 2000). The regulation mechanisms behind these two processes are totally distinct. Proliferation is dependent upon cycline-dependent kinases (CDK's), regulators of the cell cycle (Nigg, 1995), whereas caspases have a crucial role in apoptotic cell death (Nicholson, 1999). Although these are two very distinct pathways they have one common actor playing a central role, namely Ca^{2+}.

Ca^{2+} signals have an important role in the regulation of both cell proliferation and cell death by its complex signaling system (Berridge, 1995; Berridge et al., 1998). The cell transformation of a healthy cell into a tumor cell is associated with alternations in the regulatory Ca^{2+} signaling system including reorganization of Ca^{2+} pumps, Ca^{2+} channels, NCX in the plasma membrane and SR/ ER (Berridge et al., 2003; Monteith et al., 2007). Further understanding of these changes and characterization of involved actors can be very helpful in perspectives of cancer treatment.

1.4.2.1. Examples

Blood supply is required for tumor growth. This occurs via angiogenesis, a process which requires Ca^{2+} regulation (Patton et al., 2003).

CaSRs is involved in monitoring extracellular Ca^{2+} concentrations. In colon carcinoma and parathyroid hyperplasia altered levels of CaSR have been connected to dysfunctional growth suppressing effects induced by increased extracellular Ca^{2+} (Rodland, 2004). In addition CaSRs expression have been linked to proliferation in both parathyroid adenomas and carcinomas (Manning et al., 2006).

PMCA pump alteration has been associated with tumorogenesis. Overexpression of PMCA restricts Ca^{2+} in internal stores and will also contribute to faster depletion of cytoplasmic Ca^{2+} (Brini et al., 2000). Human breast cancer cell lines show expression

levels of PMCA2 that are 100-fold higher than normal (Lee et al., 2005). PMCA expression is also altered in hepatocarcinomas (Delgado-Coello et al., 2003).

Dysfunctional SERCA3 pump expression is related to colon carcinogenesis and unequal Ca^{2+} homeostasis is observed (Brouland et al., 2005). Decreased expression of SERCA2b pump is associated with increased malignancy and cell death evading is seen in the transformation of the prostate cell line LNCaP (Vanden Abeele et al., 2002).

1.4.3. Bladder cancer

1.4.3.1. Statistics and risk factors

According to GLOBOCAN worldwide cancer statistics (2008) bladder cancer is one of the 10 most commonly occurring cancer forms in Europe (9^{th} place with an incidence of 4.3 %) while being 11^{th} most common worldwide. However, it should be considered that the worldwide distribution of the bladder cancer varies greatly, with the highest incidence in industrialized countries. The incidence increase with age with a peak between 50 and 70 years and is approximately three times more frequent in males than in females (Ferlay J, 2008).

The transformation from a healthy urinary bladder epithelium to a malignancy requires some type of carcinogenic stimuli; chemical/environmental, chronic irritation or genetic. Tobacco is the major carcinogenic contributor to bladder cancer development. Tobacco represents more than 50 % of cases (Burch et al., 1989). Other causative carcinogens are; aromatic amines often present in industrial chemicals (Golka et al., 2004; Rouissi et al., 2009), ionization radiation (pelvic radiotherapy) (Kaldor et al., 1995), *Schistosoma haematobium* infection (snail fever) (Lucas, 1982) etc.

1.4.3.2. Symptoms and diagnosis

The primary symptom of bladder cancer is painless haematuria (blood in the urine). Other symptoms are changed urinary frequency, urgency and dysuria. Severe haematuria should always be further examined. Diagnosis of bladder cancer includes both urological and imaging tests including cystoscopy and biopsy. The biopsy is analyzed according to the "tumor, node, metastases (TNM) system" (Table 1.) (Sexton et al., 2010).

1.4.3.3. Treatment

The treatment choice for bladder cancer depends on many different factors including type of cancer, stage of tumor and the overall health status and age of the patient. The various alternatives of treatments available today are surgery,

immunotherapy, chemotherapy, radiation or a combination of surgery with one of the others.

Table 1. TNM Classification of Urinary Bladder Cancer (UICC 1997)

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
TIS	Carcinoma in situ
T1	Tumor invades sub-epithelial connective tissue
T2	Tumor invades the muscularis
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	microscopically
T3b	macroscopically (extravesical mass)
T4	Tumor invades any of following: prostate, uterus, vagina, pelvic wall, abdominal wall
T4a	Tumor invades prostate, uterus, or vagina
T4b	Tumor invades pelvic wall or abdominal wall
Regional Lymph Nodes (N)	
NX	Lymph node cannot be assessed
N0	No lymph node metastasis
N1	Metastasis in single lymph node, ≤ 2 cm
N2	Metastasis in single lymph node, ≥2 cm but ≤5 cm / multiple lymph nodes, ≤ 5 cm
N3	Metastasis in a lymph node, ≥ 5 cm
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

For non-muscle-invasive bladder cancer, which represents 70-80 % of the cases (Simons et al., 2008), is the initial treatment surgery i.e. transurethral resection (TUR). However, tumor recurrence occurs in up to three quarters of the patients after TUR and in some cases the tumor further progresses to invasive muscle cancer (occurs in ~20 %) (Amling, 2001). In order to prevent this recurrence and progression, non-specific immunotherapy with intravesical installation of *Bacillus Calmette-Guerin* (BCG) is used. BCG is instilled into the bladder via a catheter.

According to bladder cancer treatment many meta-analysis have been conducted from different randomized clinical trials (Reviewed by (Ayres et al., 2010; Chade et al., 2009; Gontero et al., 2010; Morgan and Clark, 2010; Shelley et al., 2010)). In summary, currently the most effective intravesical agent to treat, prevent recurrence and further progression of bladder cancer is BCG, but only if maintenance therapy is applied. Maintenance therapy is necessary since the BCG triggered immune response will reduce with time. BCG maintenance is superior to chemotherapy with for example mitomycin C (anti-tumor antibiotic that inhibit DNA synthesis) although BCG has more severe and toxic side-effects.

Two types of BCG induced side-effects can occur, either local or systematic. Local side-effects include cystitis (up to 90 % of all patients), irritating voiding symptoms, mild fever, prostatitis, haematuria. Systematic symptoms include flu-like symptoms (fever and malaise), although high fever can be connected with sepsis or hypersensitivity reaction which is the most severe side-effect as it can lead to multisystem organ failure and death (Hall et al., 2007; Rischmann et al., 2000; Sylvester, 2010; Talug et al., 2009). Both local and systematic side-effects can lead to interrupted BCG treatment. It should also be considered that not all patients will respond to BCG (BCG failures). Both withdrawal of BCG therapy and BCG failure greatly impair the prognosis.

More severe bladder cancer that has spread to the surrounding tissue generally requires cystectomy (partial or entire removal of the bladder) and metastasized tumors even more radical treatments (Sexton et al., 2010; Sharma et al., 2009).

1.4.3.4. Bacillus Calmette-Guerin

1.4.3.4.1. History of Bacillus Calmette-Guerin

Bacillus Calmette-Guerin (BCG) is a live attenuated preparation from *Mycobacterium bovis tuberculosis* (Figure 3.) which was first used as a live vaccine against tuberculosis in 1921 by Albert Calmette and Camille Guerin at the Pasteur Institute in France (Calmette, 1926).

The anticancer effect from BCG was first observed at post mortem autopsy showing a lower incidence of cancer in people treated for tuberculosis. It would take a further 50 years until the next publication about BCG in cancer treatment where it was shown that malign melanoma responded to BCG (Gutterman et al., 1973; Morton et al., 1970). However, the first effect on bladder cancer was shown in 1976. Morales et al. reported a reduction in recurrence of bladder cancer in 7 of 9 patients after intravesical treatment with BCG once a week for 6 weeks (Morales et al., 1976).

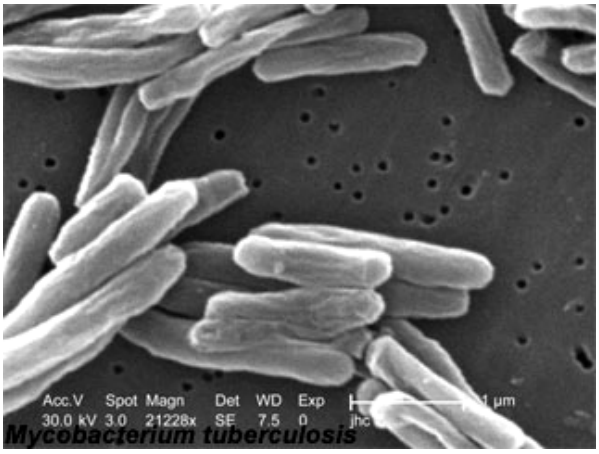

Figure 3. *Mycobacterium tuberculosis*
(http://www.lookfordiagnosis.com/mesh_info.php?term=mycobacterium&lang=6)

1.4.3.4.2. Mechanisms

BCG and its anticancer effect in bladder cancer has been closely studied over the last 35 years but the exact mechanism of action remains elusive. However it is confirmed that intravesical administration of BCG gives rise to a local immune and inflammatory response in the bladder. The response includes various immune cells (macrophages, dendritic cells, lymphocytes, neutrophiles and NK cells) and elevated levels of cytokines and chemokines that in turn may account for the anticancer effects of BCG.

Following instillation of BCG into the bladder recruitment of antigen presenting cells (dendritic cells, macrophages) will occur. Dendritic cells are the main antigen presenting cells for intracellular pathogens like BCG. BCG infected dendritic cells will release elevated levels of cytokines and chemokines that are of importance for T-lymphocyte activation (Demangel and Britton, 2000). They will process BCG, release inflammatory cytokines/chemokines and present BCG for T-lymphocytes.

Different reports support the theory that BCG-infected dendritic cells lead to T-lymphocyte activation and elevated levels of IL-2, IL-8, IL-10, IL-12, INF-γ and TNF-α (Cheadle et al., 2003; Higuchi et al., 2009; Ramoner et al., 1998). This activation is dependent on presence of $CD4^+$ and $CD8^+$ T-lymphocytes to mediate the anticancer activity (Ratliff et al., 1993).

This will in turn attract and activate other immune cells like γδT-lymphocytes and natural killer cells and neutrophiles (Naoe et al., 2007). γδT-lymphocytes represent a small group of T-lymphocytes expressing T-cell receptor (TCR) and are involved in both innate and adaptive immune responses while natural killer T cells are a subpopulation of T-lymphocytes expressing both NK cell markers and T-cell receptors and are primarily involved in the detection of bacterial and parasitic

pathogens (Munz et al., 2005). One hypothesis is that the elimination of tumor cells occurs through γδT-lymphocytes and natural killer T cells (via a pathway including stress-associated tumor specific MICA/MICB molecules and the NKG2D receptor) (Higuchi et al., 2009).

TNF-related apoptosis inducing ligand (TRAIL), one molecule able to induce apoptosis in neoplastic but not healthy cells, is expressed and secreted from neutrophiles following BCG stimulation and seems to contribute to the anticancer effect (Kresowik and Griffith, 2009; Simons et al., 2008).

Although the immune response is of great importance, perhaps even essential, for the anticancer effect, the direct interaction between BCG and the tumor should not be disregarded. It is possible the direct interaction might be crucial to trigger the immune response.

BCG can interact directly with the tumor cells which is followed by internalization and degradation of BCG (Becich et al., 1991). *In vitro* studies have reported that BCG increases the expression of intracellular adhesion molecule (ICAM)-1, a molecule required for linking tumor cells with immune cells (T lymphocytes and neutrophiles) (Alexandrov AV, 1997; Jackson et al., 1994). Elevated levels of IL-6, IL-8, IL-16, GM-CSF IFN-α, INF-γ have also been reported (Esuvaranathan et al., 1995; Zhang et al., 1999). The IL-6 production might be regulated through a cAMP pathway and requires NF-κB activation (Chen et al., 2002; Zhang et al., 2002). In addition *in vivo* studies have reported amplified levers of IL-1, IL-6 and TNF-α due to BCG treatment (Esuvaranathan et al., 1995; Sander et al., 1996).

Furthermore, introduction of BCG suppresses proliferation of bladder cancer cell in a dose and time dependent mode (Jackson, 1994) and induces caspase independent cell death with necrotic characteristics, including release of a typical necrosis marker protein (high molecular group box protein 1) (See et al., 2009). In contrast to the last study two other studies have reported that BCG itself is not able to induce cell death (Chen et al., 2007; Sasaki et al., 1997). In our current study we have shown that direct interaction between BCG and human bladder cancer cell line T24 results in reduced proliferation via PLC (unpublished data, paper III).

On the other hand it should be considered that it is not established if a healthy bladder epithelium will response in the same way as a tumor cell or not.

1.4.3.4.3. Further perspectives

Today the most prominent treatment of bladder cancer is BCG even if it has been hardly challenged with newer agents. However all available treatments including

BCG have serious limitations and needs to be improved. One of the most serious drawbacks with BCG is the severe side-effects. There are patients that cannot continue the treatment due to side-effects (around 20 %) because of the high toxicity of BCG. The massive release of cytokines and chemokines during the immune response might be of big contribution to the side-effect. To minimize the side-effects the optimal dose of BCG, the induction time and duration of the treatment need to be determined, although optimal treatments will vary from patient to patient. Deeper understanding of the anticancer effect and supplementary treatment with other agents might be alternative solutions. The failure of some patients to respond to BCG treatment is yet another drawback which will need to be resolved.

1.5. Maintenance of membrane potential

There is an electrical potential or voltage across the plasma membrane in all living cells. This voltage is referred to as the resting membrane potential and is a consequence of unequal distribution of ions outside and inside the cells, the membrane permeability for those ions (i.e. ion conductions through ion channels) and active ion pumps. There is usually a higher concentration of potassium (around 20 times higher) and different anions inside the cell and higher concentrations of sodium (about 10 times higher) and chloride outside the cell. This ion distribution gives, for example an excitable cell like a neuron a net negative charge and a resting membrane potential around -70 mV when it is not producing an action potential in neurons, muscle and heart cells is the resting membrane potential the driving force for communication and other processes (Wright, 2004).

Two factors that are involved in the maintenance of the membrane potential are: *i*) the sodium pump or Na^+,K^+-ATPase and *ii*) hyperpolarization-activated cyclic nucleotide-gated channels.

1.5.1. The sodium pump and its functions

1.5.1.1. The sodium pump

Na^+,K^+-ATPase or the sodium pump was first described in 1957 by Jens C Skou (Skou, 1957). In 1997 Jens C Skou was rewarded with the Nobel Prize in chemistry for his finding. The sodium pump is located in the plasma membrane in all mammalian cells where it transports cations against their concentration gradient; 3 Na^+ out and 2 K^+ in utilizing energy from one ATP. This process stands for around 30 % of the entire energy consumption in a cell. The pumping generates an electrical gradient across the plasma membrane and thereby making the sodium pump responsible for establishing and maintaining the electrochemical gradient and resting potential in cells (Scheiner-Bobis, 2002; Skou and Esmann, 1992). This gradient is essential for the excitability in muscles and brain cells, furthermore it drives many transport mechanisms of ions, amino acids, vitamins. Apart from this the sodium pump plays a fundamental role in many biological processes and has a very complex function.

1.5.1.2. Structure of the sodium pump

The sodium pump belongs to the same family as the PMCA and SERCA pumps, namely the P-type ATPase super-family (Lutsenko and Kaplan, 1995). The sodium pump consists of two main subunits, α & β and depending on tissue sometimes also a third, γ. The α subunit is a multispanning membrane protein that possesses the catalytical function of the enzyme and accounts for the transport of Na^+ and K^+. It exists in four isoforms (α1-4). The β subunit, a polypeptide, is the regulatory part of

the enzyme and is necessary for proper protein folding and integration into the plasma membrane. It exists in three isoforms (β1-3) (Blanco and Mercer, 1998; Kaplan, 2002; Sweadner, 1989). The γ subunit (an auxiliary subunit, belonging to the FXYD family) exist in some tissues where it modulates the enzymatic activity but it is not required for the functional enzyme activity (Geering, 2005; Geering, 2006). The presence or absence of the γ subunit is not fully established.

Both α and β subunits are expressed in a tissue specific manner. The α1 isoform is ubiquitously expressed while the α2 isoform expression is restricted to skeletal, heart and smooth muscle, brain, lung and adipocytes. The α3 isoform is mainly found in neurons and ovaries and α4 isoform is expressed in sperm (Kaplan, 2002; Lingrel and Kuntzweiler, 1994; Sweadner, 1989; Woo et al., 1999).

1.5.1.3. Cardiotonic steroids

Cardiotonic steroids (CTS) are a group of compounds naturally found in plants and including digitoxin, digoxin and around 250 other compounds (Krenn and Kopp, 1998). Digitalis is also known as digoxin, an extract from *Digitalis purpurea* (Foxlove) (Figure 4.), which has been used to treat arrhythmias and congestive heart failure for more than 200 years.

CTS acts by binding to the extracellular loops of α subunit in the sodium pump inducing a conformational change and thereby inhibiting the pump in a dose-dependent manner.
This leads to an increase in the intracellular Na^+ concentration which in turn elevates the intracellular concentration of Ca^{2+} via the Na^+/Ca^{2+}-exchanger which in turn increases the cardiac output (Blaustein, 1993).

Figure 4. *Digitalis purpurea*

In 1991 Hamlyn and coworkers reported the occurrence of endogenous ouabain in human plasma (Hamlyn et al., 1991). Thereafter endogenous ouabain or ouabain-like material has been found in the bovine adrenal gland and bovine hypothalamus (Schneider et al., 1998; Tymiak et al., 1993) and most recently in rat adrenomedullary cells (Komiyama et al., 2001). Mass spectroscopy and nuclear magnetic resonance experiments have reported that the endogenous ouabain is identical to the plant-derived ouabain. It is considered that the endogenous ouabain is synthesized in the adrenal gland and in the hypothalamus even if the total process has not been identified (el-Masri et al., 2002; Komiyama et al., 2001; Murrell et al., 2005). Endogenous ouabain has hormonal functions and has been established to

have similar function as CTS i.e. binding and inhibiting the sodium pump in a dose-dependent manner. Distinct from other proteins ouabain are not able to penetrate the plasma membrane.

The amino acid sequence of the CTS binding site is highly conserved during evolution suggesting an important biological function. However, the affinity for CTS varies between species but also among the different isoforms of the α subunit for unknown reasons. All human α isoforms are sensitive to CTS and have similar K_d values in nM range while in, for example mouse and rat, only isoforms α2-α4 are sensitive whereas α 1 is insensitive to ouabain (Crambert et al., 2000). Data has demonstrated that two amino acids alterations in position 111 and 122 are responsible for the insensitivity for ouabain in mouse and rat α1 isoform (Price and Lingrel, 1988).

Elevated levels of endogenous ouabain have been found in animals and humans due to physical exercise (Bauer et al., 2005; Goto et al., 1995) and *in vitro* studies have shown that secretion of ouabain from the adrenal cortex is stimulated through angiotensin II, phenylephrine and vasopressin (Laredo et al., 1997; Shah et al., 1999).

It has been questioned if endogenous ouabain acts as a natriuretic hormone however it does not fulfill all criteria's even if it is involved in both sodium loading and depletion. There are studies with conflicting results both disagreeing and promoting ouabain's role in the regulation mechanism of blood pressure. Ho *et al*. and Butt *et al*. have both showed that intake of salt will stimulate ouabain secretion in rats (Butt et al., 1997; Ho et al., 1997). In contrast Manunta *et al*. showed that salt intake over 2 weeks did not affect the plasma levels of ouabain in a group of patient with untreated hypertension. However 2 weeks with salt depletion did increase ouabain levels. (Manunta et al., 2001). Balzan et al has also reported that ouabain levels are not altered due to salt intake (Balzan et al., 2005). They have also reported that ACTH is a possible factor able to modulate ouabain secretion during hypertension. Furthermore elevated levels of ouabain has been reported under ACTH-induced hypertension (Goto et al., 1996). Prolonged administration of ouabain in two groups of mice, one expressing an ouabain-insensitive α2 isoform in the sodium pump and the other expressing an ouabain sensitive α2 isoform, showed increased levels of ouabain in both groups but only the group with the ouabain-sensitive α2 isoform developed high blood pressure, suggesting a role for ouabain in blood pressure (Dostanic et al., 2005). The cardiotonic steroid-binding site of the α2 isoform also plays a role in maintaining normal systolic blood pressure during pregnancy (Oshiro et al.).

1.5.1.4. Signal transduction

More recently it has been established that the sodium pump in addition to be an energy-transducing ion pump can transmit signals and activate different intracellular pathways. However, it is when the CTS concentration are so low that it is insufficient to inhibit the pump and alter intracellular Na^+ and K^+ concentrations that CTS will contribute to signal transduction via activation of intracellular signaling pathways.

Xie and coworkers have, in a series of publications (Xie, 2001; Xie and Askari, 2002; Xie and Cai, 2003; Xie and Xie, 2005; Zhang et al., 2008), shown that Src, a small membrane-associated non-receptor tyrosine kinase, interacts with the sodium pump in the caveolae (Tian et al., 2006; Wang et al., 2004). Binding of ouabain to the sodium pump furthermore activates Src which results in tyrosine phosphorylation of multiple effectors and thereby initiates different signaling. This also includes transactivation of epidermal growth factor receptor (EGFR) (Haas et al., 2002).

Further downstream events of the sodium pump mediated signal transduction can be *i*) recruitment of the adoptor protein Shc that will activate the Grb/Ras/Raf/MEK/ERK pathway (Haas et al., 2000) *ii*) activation of PLC that will result in production of $InsP_3$ and DAG (Puceat and Vassort, 1996) or *iii*) mitochondrial production of ROS (Xie et al., 1999).

Moreover it has been established that binding of ouabain to the sodium pump activates slow Ca^{2+} oscillations which further cause activation of the transcription factor NF-κB in renal epithelial cells (Aizman et al., 2001). Later it was shown that the sodium pump interacts with the $InsP_3R$ forming a microdomain. Binding of ouabain to the sodium pump affected this interaction by altering the conformation of the pump which results in the Ca^{2+} oscillations (Miyakawa-Naito et al., 2003). Further it has been demonstrated that the interaction between the sodium pump and the $InsP_3R$ occurs in the N-terminal part of both proteins and that the adaptor protein ankyrin-B also interacts in the same region (Chen et al., 2008; Kline et al., 2008; Liu et al., 2008). The ouabain induced Ca^{2+} oscillations have also been shown to be involved in the rescue of cells from serum-deprivation-induced apoptosis (Li et al., 2006).

In summary the great diversity in function of the sodium pump is partially a result of the wide variety of isoforms for the subunits and partially a result of that the interaction between CTS and it binding site in the sodium pump can result in diverse functions depending on the concentration of the CST (Figure 5.).

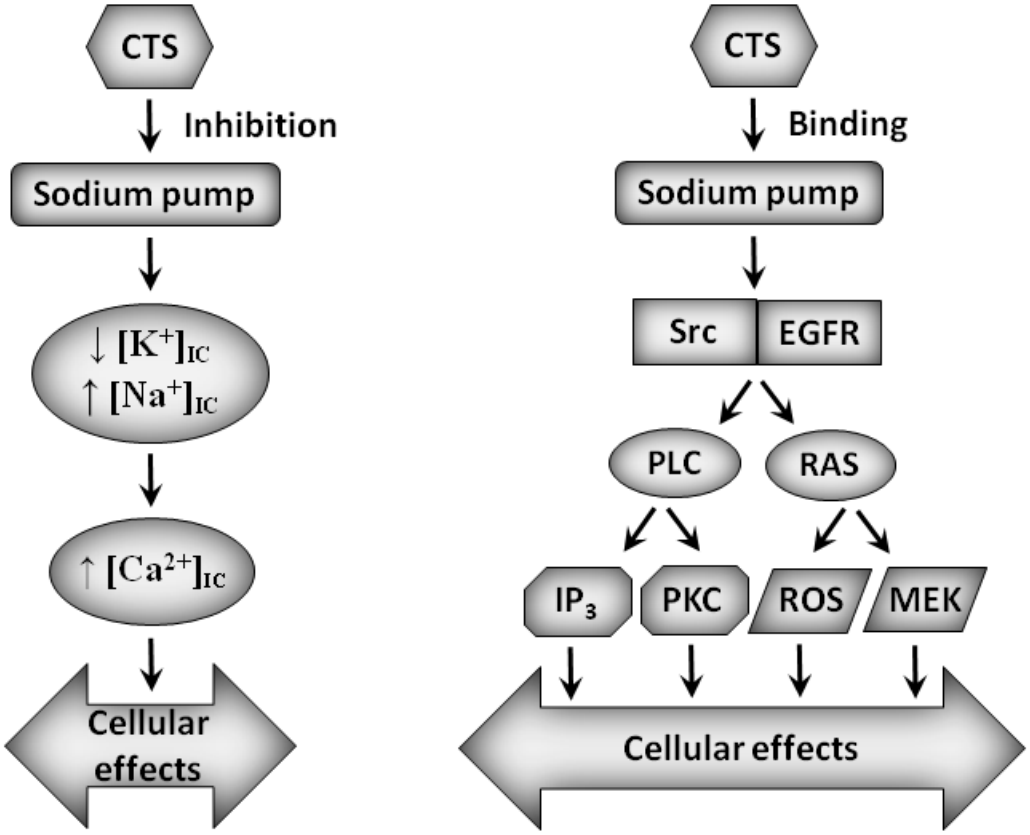

Figure 5. Schematic draft over two pathways mediated by ouabain and the sodium pump

1.5.2. Hyperpolarization-activated cyclic nucleotide-gated channels

1.5.2.1. Hyperpolarization-activated cyclic nucleotide-gated channels

Rhythmic electrical activity is found both in the heart and the nervous system. In the heart this electrical activity is an inward current of cations I_f (f=funny current) seen in "pacemaker" cells which controls the commencement and modulation of the heart beat. In distinct types of neurons a similar current has been identified; I_h (h=hyperpolarization current). This current is mainly involved in the regulation of the rhythmic activity in the neuronal circuit but also in other processes such as: regulation of the resting membrane potential, synaptic transmission, neuronal development including dendritic integration and controlling spontaneous Ca^{2+} oscillations and processing of temporal signals (Biel et al., 2009; Craven and Zagotta, 2006). The responsible actor for those currents has been identified as hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and are members of the voltage-gated cation channel superfamily (Jackson et al., 2007).

1.5.2.2. Structure

The HCN channels are build up of six trans-membrane helical segments (S1-S6) including a positively charged segment (S4) acting as voltage sensor (Chen et al., 2000), an ion conducting pore (between S5 and S6) (Jackson et al., 2007) and finally a c-terminal α-helical linker region that joins the trans-membrane region to a highly evolutionary conserved cyclic nucleotide binding domain (CNBD) (Figure 6.) (Biel et al., 2002; Zagotta et al., 2003). The C-linker-CNBD is an autoinhibitory domain that in the absence of cAMP reduces the opening probability of the channel (Biel et al., 2009). Each HCN channel consists of homo- or heterotetramers arranged so the ion conducting pore will be located in the central part of the channel (Biel et al., 2009; Craven and Zagotta, 2006).

In mammals there exist four different HCN channel subunits, HCN channel 1 – 4. They are normally expressed in the heart and the nervous system but the expression pattern among the different subunits differs. Generally HCN channel 1, 2 and 4 are found in brain and heart while HCN channel 3 is mainly found in neurons (Ishii et al., 1999; Ludwig et al., 1998; Santoro et al., 1998).

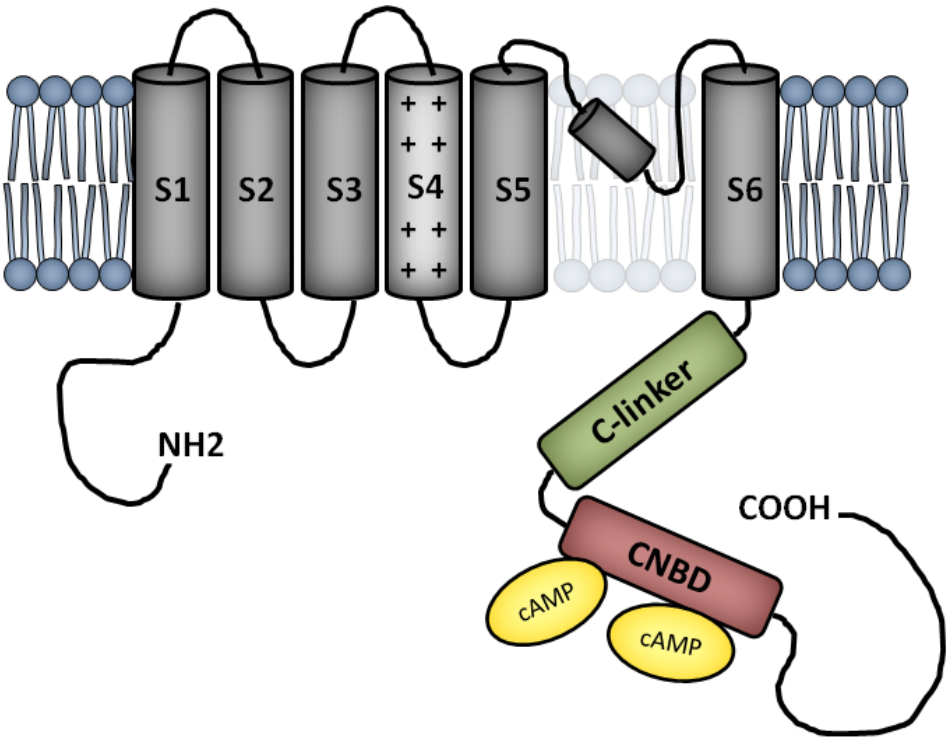

Figure 6. Structure of the HCN channel

1.5.2.3. Basic biophysical properties

HCN channels present specific biophysical characteristics:

1. Channel activation by membrane hyperpolarization.
2. Channel activation is facilitated by direct interaction of cyclic nucleotides to the CNBD.
3. Permeable to Na^+ and K^+
4. Channel inactivation by Cs^+

1.5.2.3.1 Hyperpolarization

The HCN channels are activated by membrane hyperpolarization. The $V_{0.5}$ values (membrane voltage at 50 % of maximal charge movement) as well as the activation kinetics for voltage-dependent activation differ among the four HCN channels. For example the HCN channel 1 has the fastest kinetics whereas HCN channel 4 has the slowest properties (Biel et al., 2009).

1.5.2.3.2. Cyclic nucleotides

An elevated level of cAMP facilitates activation of the channel by shifting the $V_{0.5}$ value towards a more positive value and thereby accelerates the opening kinetic of the channel. Consequently high concentration of cellular cAMP will result in a channel opening that is faster and more complete compared to that at low cAMP

concentration. Thus is the cAMP regulation of HCN channels of great importance when it comes to physiological mechanisms since the channel are responsible for the electrical (e.g. membrane potential) and chemical (e.g. neurotransmitter and hormones) contribution. cGMP are equivalent in function to cAMP although the affinity is different and a 10 fold higher concentration of cGMP is needed to receive the same effect in shifting the potential towards a more depolarized voltage as for cAMP (Biel et al., 2009; Craven and Zagotta, 2006; DiFrancesco and Tortora, 1991; Ludwig et al., 1998).

1.5.2.3.3. Permeabilization

HCN channels are permeable to K^+ and Na^+ (DiFrancesco, 1993; Ludwig et al., 1998; Santoro et al., 1998; Yu et al., 1995). They are slightly more selective for K^+ over Na^+ (ration 4:1) which will result in an inward ion current of Na^+ that will depolarize the membrane toward the threshold to trigger an action potential due to activation of the channel at resting membrane potential (around -70 mV) (Craven and Zagotta, 2006).

In addition to K^+ and Na^+ two of the channels, HCN channel 2 and 4, are also permeable to Ca^{2+} (Michels et al., 2008; Yu et al., 2004). The physiological implication of Ca^{2+} signaling through these channels has not been clear. However a role for the HCN channel 2 in apoptosis signaling was recently suggested. Kinase inhibitors (staurosporine and PKC412) can causes a Ca^{2+} influx through the HCN channel 2 through phosphorylation of Thr^{549} within the internal regulatory domain of the channel. The imported Ca^{2+} then triggers apoptosis mediated by calpain and AIF (Norberg et al., 2010a).

1.5.2.3.5. Inactivation

One other characteristic of those channels include a pharmacological outline showing inactivation of the channels with milimolar concentration of Cs^+ although they are unaffected of Ba^{2+} (Biel et al., 2009; Craven and Zagotta, 2006).

1.5.2.4. Further regulations mechanisms of the channels

Wu and Cohen suggested in late the 1990's that HCN channels can be regulated through tyrosine kinases from the Src family (Wu and Cohen, 1997). Their founding was supported by mapping experiments that demonstrated that Src actually binds to the C-linker-CNBD in HCN channel 2 via its SH3 domain and thereby phosphorylates the channel (Zong et al., 2005). A consequence of this phosphorylation is accelerated channel activation. It has been further demonstrated that HCN channels can be regulated by tyrosine phosphorylation via Src kinases under physiological conditions

in pacemaker cells in the sinoatrial node in mice and rat heart as well as in neurons (Biel et al., 2009).

Another regulation mechanism of the HCN channels is via p38-mitogen-activated protein (MAP) kinases. If the activation occurs directly via phosphorylation the channel or indirectly via phosphorylation of another protein interacting with the channel is not established (Poolos et al., 2006). Different experiments have demonstrated that activation via p38-MAP kinase in hippocampal pyramidal neurons shifts the voltage dependent activation potential in the direction of a more positive value and thereby influences neuronal excitability and temporal summation (the way in which non-simultaneous synaptic events combine in time) (Biel et al., 2009).

1.5.2.5. Pathology

HCN channels are expressed in the nervous system and heart and channel defects have been related with diseases such as epilepsy, neuropathic peripheral pain and cardiac arrhythmia. Cardiac hypertrophy and heart failure are consequences of upregulated I_f as a result of enhanced expression of HCN channels. It has been shown that upregulated I_f results in ectopic pacemaker like patterns in the ventricular muscle that can lead to arrhythmia and heart failure (Biel et al., 2009).

2 Aims

The different projects in this thesis investigated the role of Ca^{2+} in different developmental and disease processes. Further defined aims were:

1. Examine the impact of Ca^{2+} and Na^{+}, K^{+}-ATPase signaling in dendritic growth in cortical neurons

2. Characterize the mechanism behind the prolonged Ca^{2+} increase following treatment with PKC inhibitors (staurosporine and PKC412).

3. To investigate how Ca^{2+} homeostasis was affected by BCG in human bladder cancer (T24) cells and to look at downstream targets.

3 Results and discussion

3.1 Paper I – Na^+, K^+-ATPase signal transduction triggers CREB activation and dendritic growth

Neuronal cells are characterized by dendrites that form the complex signaling network for different functions in the brain. The growth of dendrites includes a dynamic process taking place during neurogenesis accomplice by a variety of factors.

3.1.1. Na^+, K^+-ATPase receptor activation triggers dendritic growth in cortical neurons

To investigate the impact of Na^+, K^+-ATPase or the sodium pump on dendritic growth primary cultures of rat cortical neurons were stimulated with 1 µM ouabain for 48 hours. Received data showed an enhanced dendritic growth with respect to both length and number compared with the mock treatment. The dendritic lengths were nearly 2-fold increased and the number of dendrites improved by almost 100 %.

3.1.2. Na^+, K^+-ATPase signal transduction occurs without changing the resting membrane potential

Next $^{86}Rb^+$ uptake experiments were performed to rule out the inhibitory effect of ouabain on the Na^+, K^+-ATPase. These data showed that 1 µM ouabain only inhibited the pump by 20.7 ± 4.6 % and that the IC_{50} for cortical neuron Na, K-ATPase was 13.2 µM for ouabain. These findings suggest that the Na^+, K^+-ATPase conveys the effect due to signal transduction rather than pump inhibition. Since Na^+, K^+-ATPase primary role is to establish the electrochemical gradient in cells (Kaplan JH 2002), ouabain's effect on the resting membrane potential was examined by whole-cell patch clamp recordings. Ouabain 1 µM did not have any significant effect on the resting membrane potential while ouabain 100 µM depolarized the membrane. However, ouabain 100 µM had no effect on dendritic growth so membrane depolarization alone was not sufficient to trigger dendritic growth. These results indicate that the concentration of ouabain used (1 µM) neither inhibits the pump nor depolarizes the cells.

In addition Ca^{2+} oscillations were observed following ouabain treatment for 6 hours. Furthermore the L-type Ca^{2+} blocker nifidepine inhibited the Ca^{2+} response.

3.1.3. MAP kinase phosphorylation is stimulated by Na^+, K^+-ATPase signaling

MAP kinases are serine/threonine-specific protein kinases that have been shown to be involved in the regulation of dendritic growth and determination of neuronal

morphology. To look at the involvement of MAP kinases in Na^+, K^+-ATPase signal transduction phosphorylation of ERK 1/2 (p42/44 MAP kinase) was monitored by Western blotting. Cortical neurons stimulated with ouabain for 6 hours showed a sustained ERK 1/2 phosphorylation. This phosphorylation was eliminated by the upstream MEK inhibitor U0126. To investigate the effect of Ca^{2+} on ERK 1/2 phosphorylation cells were pre-incubated with nifedipine, which reduced the phosphorylation level. The ERK 1/2 phosphorylation was furthermore partially abolished by the CaM kinase inhibitor KN93.

3.1.4. Na^+, K^+-ATPase signal transduction triggers CREB transcription through CaM kinases

One transcription factor implicated in neuronal differentiation which is regulated via CaM kinase is CREB. To study the involvement of CREB immunocytochemistry experiments were performed. Six hours ouabain treatment showed increased levels of Ser-133 phosphorylated CREB. These results were further supported by Western blot. Next the pathways involved in the Na^+, K^+-ATPase signal transduced CREB phosphorylation was further examined. The MEK inhibitor U0126, CaM kinase inhibitor KN93 and L-type Ca^{2+} blocker all reduced the levels of Ser-133 phosphorylated CREB. Even if phosphorylation of Ser-133 is required for CREB-dependent transcription it is not adequate for gene activation (Wayman et al 2006). To resolve this problem a CRE-luciferase assay was done. These data showed a 3 fold increase in CRE activity after 6 hours treatment with ouabain and the CRE activity were totally abolished by KN93.

3.1.5. Dendritic growth induced by Na^+, K^+-ATPase signaling is orchestrated by MAP kinases, CaM kinases and extracellular $Ca2^+$ influx

Finally, the involvement of Ca^{2+}, MAP kinases and CaM kinases on Na^+, K^+-ATPase signal-transduction induced dendritic growth was investigated. The Ca^{2+} inhibitor nifedipine and the MEK inhibitor U0126 both significantly decreased the neurite outgrowth while the CaM kinase inhibitor KN93 completely abrogates the outgrowth.

3.1.6. Conclusion

In this study we demonstrated that Na^+, K^+-ATPase signal transduction is able to stimulate dendritic growth in cortical neurons from rat. This biological process activates a transcriptional program involving activation of CREB and CRE regulated via the CaM kinase pathway, sustained MAP kinase phosphorylation and intracellular Ca^{2+} oscillations (Figure 7). These findings suggest a novel role for Na^+, K^+-ATPase as a modulator of dendritogenesis in developing cortical neurons in the brain and that it therefore might play an important role in circuit wiring.

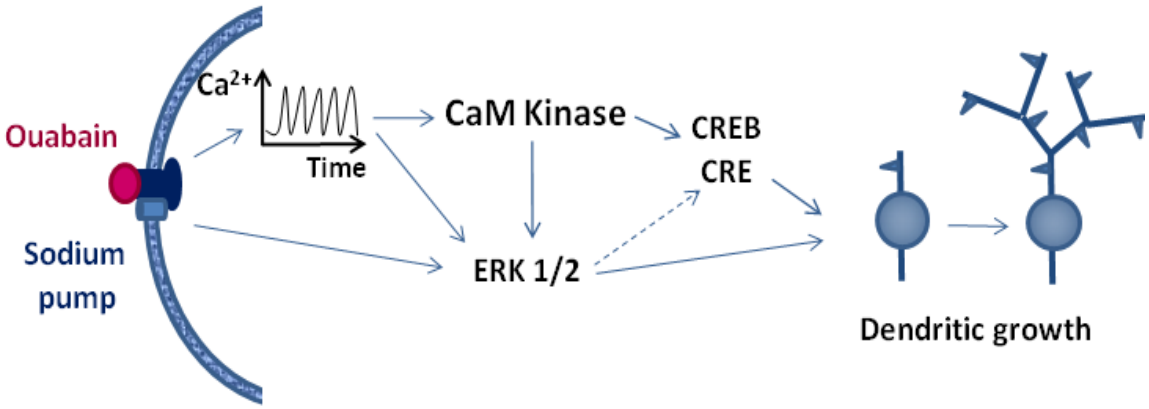

Figure 7. Schematic draft over Na^+, K^+-ATPase signal transduction and dendritic growth

3.2 Paper II – Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis

Non-small-cell lung carcinomas (NSCLC) are resistant to conventional cancer therapy. However, the protein kinase C (PKC) inhibitors staurosporine (STS) and PKC412 are both able to trigger cell death in those cells (Josephs et al. 2002, Gallego et al. 2004). It has been previously reported that these PKC inhibitors induces cell death through a prolonged elevation in the intracellular Ca^{2+} from the extracellular milieu (Norberg et al. 2008).

3.2.1. PKC inhibitor induced Ca^{2+} influx occurs via hyperpolarized-activated cyclic nucleotide-gated channel 2 in NSCLC cells

To further investigate the involvement of Ca^{2+} and which channels that might be responsible for the Ca^{2+} elevation, different antagonists of the voltage-dependent Ca^{2+} channels (VDCCs) was tested but none had any effect on the Ca^{2+} influx. Most VDCC are activated by depolarization and only a few by hyperpolarization (Siegelbaum et al 2000). Thus, STS and PKC412 ability to affect the membrane potential was examined. Unexpectedly a hyperpolarization of the cells was detected with both STS and PKC412.

As mentioned before only a few plasma membrane channels are activated by hyperpolarization. The criteria that they also need to be permeable for Ca^{2+} make the list of choice even shorter. Interestingly, a family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are activated by hyperpolarization and two of four channels, HCN channel 2 and 4, are furthermore permeable to Ca^{2+} in addition to K^+ and Na^+ (Yu et al. 2004, Michels et al 2008).

Therefore, the possibility of involvement of HCN channels was further studied. Cells were treated with Cs^+ before exposure of STS or PKC412 meanwhile recording the membrane potential and Ca^{2+} levels. Cs^+ treatment was shown to inhibit both the hyperpolarization and the Ca^{2+} elevations. Suggesting that hyperpolarization is required for the Ca^{2+} influx, and that this most probably occurs through the HCN channels.

Next the expression levels of HCN channel 2 and 4 in U1810 and H661 cells was investigated. mRNA expression was noted for both HCN channel 2 and 4 while protein expression only was detected for HCN channel 2. To further investigate the potential role of HCN channels in the Ca^{2+} influx, HCN channel 2 and HCN channel 4 was downregulated by siRNA and intracellular Ca^{2+} levels was recorded while exposing the cells for STS or PKC412. Interestingly, downregulation of HCN channel 2 but not HCN channel 4 was shown to eliminate the STS/PKC412 induced Ca^{2+} influx.

3.2.2. Ca^{2+}influx through HCN channel 2 leads to AIF-mediated cell death

Previously STS and PKC412 have been shown to induce elevated levels of intracellular Ca^{2+} that can trigger apoptosis in NLCSC cells (Norberg et al. 2008). So next, it was investigated if the STS/PKC412 induced Ca^{2+} influx via HCN channel 2 was adequate to trigger apoptosis in this case. These results showed that the Ca^{2+} entry through HCN2 is sufficient to induce apoptosis.

The mechanism that triggers cell death was additional examined. Cleavage of Atg5 and AIF (two selective calpain substrates) was monitored in control cells but were suppressed in cells with downregulated HCN channel 2. These results were supported by flow cytometry analysis of Annexin V staining, counting of condensed nuclei, processing/activation of caspase-2,-3,-8,-9 and monitoring of PARP cleavage and finally caspase-3, -like activity measurements. All demonstrating that HCN channel 2 induced Ca^{2+} influx triggers a caspase-independent, AIF-mediated apoptosis.

Another cell line, HEK29, which normally lacks the HCN channels, is therefore commonly used as a model system to study the heterologus HCN channel expression (Yu et al 2004). It was shown that stimulation with STS/PKC412 could not as expected induce a Ca^{2+} influx in those cells. However, after introducing the HCN channel 2 channel into the cells a prolonged Ca^{2+} influx could be monitored. Similar to earlier results, AIF was released from the mitochondria due to STS exposure, only in cells expressing HCN channel 2. Flow cytometry analysis with Annexin V staining demonstrated that HEK293 cells survived STS/PKC412 treatment at least 24 h while expression of HCN2 sensitized the cells to the STS/PKC412 treatment. These data demonstrated that HCN2 was indeed responsible for cellular Ca^{2+} influx after treatment with PKC inhibitors.

3.2.3. Dephosporylation of THr^{549} is fundamental for the STS/PKC412 induced Ca^{2+} influx through HCN channel 2

cAMP and phosphorylation are two critical events for opening the HCN channels (Zong et al 2003, Biel et al 2009). To further look at the phosphorylation mechanism, cells were exposed to two PKC activators before stimulated with STS/PKC412. The results here showed that the PKC activators instead of giving a prolonged Ca^{2+} elevation gave a fast Ca^{2+} peak. However, Norberg et al 2008 have shown that this short Ca^{2+} peak is inadequate to trigger apoptosis.

Analysis of the Ser/Thr phosphorylation level of the HCN channel 2 in open/closed states showed increased phosphorylation in control cells while STS exposure dephosphorylated the channel while the PKC activators were able to suppress this decrease.

In silico analysis of the HCN channel 2 exposed four PKC phosphorylation sites within the internal C-terminal domain of the channel. Alignment showed that these phosphorylation sites are conserved between human and mouse. Thereafter site-directed mutagenesis of the four phosphorylation sites where Ser/Thr was substituted to an Ala was performed. The mutants showed a similar Ca^{2+} response to STS/PKC412 as the wild-type (non-mutated).

As previously described STS/PKC412 triggers activation of the HCN channel 2 via hyperpolarization and dephosphorylation. Therefore, the Ala might be opened by the same mechanism or due to the hyperpolarization. Experiments with reduced levels of K^+ showed a hyperpolarization in the wild-type as well in all mutants while a prolonged Ca^{2+} response only were observed with the Thr^{549} mutant. Proposing that dephosporylation of the residue Thr^{549} is crucial for the prolonged Ca^{2+} response.

3.2.4. STS/PKC412 induces a prolonged Ca^{2+} influx via HCN channel 2 also in cortical neurons

In vivo data proposed that the AIF-mediated pathway might be critical for neuronal cell death (Zhu et al 2007a, 2007b, Hangen et al 2010). Therefore, it was investigated if a similar mechanism seen in carcinoma lung cells was also observed in primary cultures from rat cortex. Both STS and PKC293 induced a Ca^{2+} influx which was abolished by Cs^+ and depletion of HCN2. HCN2 depletion also prevented the AIF processing as well as suppressed the amount of condensed nuclei.

3.2.5. Conclusions

Here we have showed that the PKC inhibitors STS and PKC412 both can trigger a Ca^{2+} influx through the HCN channel 2 is adequate to trigger AIF-mediated apoptosis in lung carcinoma cells and primary culture of cortical neurons (Figure 8.). Furthermore, down-regulation of the HCN channel 2 prevented the Ca^{2+} influx and the following apoptosis. In HEK293 cells, not expressing the HCN channels the PKC inhibitors did not cause any Ca^{2+} influx or apoptosis. However, expression of HCN channel 2 sensitized them to STS/PKC412 induced apoptosis. Finally, mutagenesis of PKC phosphorylation sites at the C-terminal domain of the HCN2 revealed that dephosporylation of Thr^{549} was critical for the Ca^{2+} influx required for AIF-mediated apoptosis. These results demonstrate a novel role of the HCN channel 2 as an upstream regulator of cell death triggered by PKC inhibitors. This finding might have an important role in the treatment of NSCLC. Since the HCN channel 2 is a potential drug target the expression pattern of HCN channel 2 will be analyzed in patient samples.

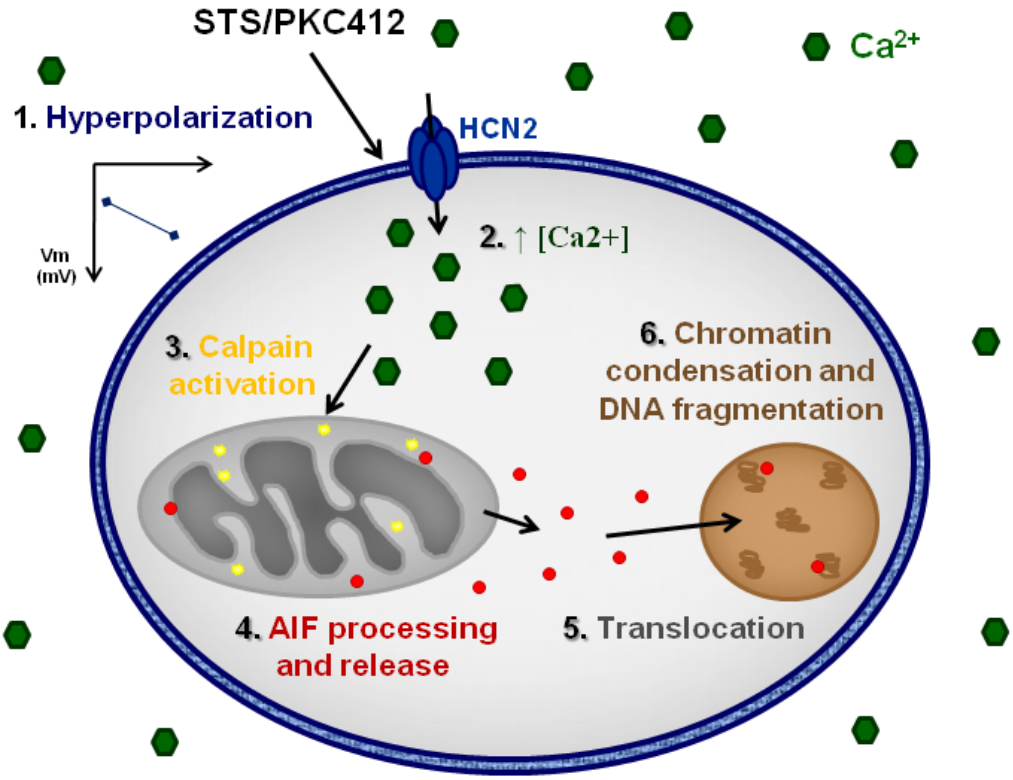

Figure 8. Schematic cartoon over STS/PKC412 induced AIF apoptosis

3.3 Paper III – Bacillus Calmette-Guerin (BCG) Stops Urinary Bladder Cancer Cell Proliferation through PLCβ3-Dependent Ca^{2+} Signaling

Bladder cancer refers to malignant growth in the urinary bladder. 70-80 % of bladder cancer is non-muscle invasive cancer (Simons et al., 2008). The initial treatment for this type of cancer is transurethral resection (TUR) followed by immunotherapy with *Bacillus Calmette-Guerin* (BCG). BCG triggers an immune response that recruits different immune and inflammatory cells and elevated level of chemokines and cytokines in the bladder. However the exact mechanism is not fully established.

3.3.1. BCG induces an intracellular Ca^{2+} response in human urinary bladder cells

Our main purpose was to examine a more direct effect of BCG on cancer cells. To investigate how Ca^{2+} homeostasis was affected by BCG, human bladder cancer cells (T24) were loaded with the Ca^{2+} sensitive fluorescent dye Fluo-3/AM. Those experiments showed a Ca^{2+} response (including oscillations, transients and sustained Ca^{2+} responses) in both presence and absence of extracellular Ca^{2+} indicating an intracellular source of Ca^{2+}. Furthermore, addition of peptidoglycan (PGN) (a polymer from the bacterial cell wall) to the cells triggered a Ca^{2+} response similar to the one from BCG.

The intracellular localization of internalized BCG was studied by using BacLight stained BCG, a bacterial dye staining the bacterial membrane, which were monitored using confocal microscopy. Double-staining with ER-tracker suggest a co-localization between BCG and ER. After internalization of BCG to the cell it might interact with ER but this needs further investigations.

3.3.2. PLC is affecting BCG induced Ca^{2+} singling

Next pharmacological studies were performed to investigate downstream targets of BCG. Pretreatment with Cyclopiazonic acid (CPA), an inhibitor of the sarcoplasmic/endoplasmic reticulum calcium (SERCA) pump, reduced the Ca^{2+} response triggered by BCG. These data support the proposal that internal stores represent one Ca^{2+} source in this pathway. The G-protein coupled receptor inactivator Pertussis toxin (PTX) also suppressed the Ca^{2+} increased.

One pathway of generating intracellular Ca^{2+} release is via PLC which processes PIP_2 to $InsP_3$ and diacylglycerol (DAG). $InsP_3$ in turn stimulates Ca^{2+} release via $InsP_3R$. U73122 and ET-18-OCH_3, two PLC inhibitors, significantly suppressed the Ca^{2+} response; however U73122's inactive analogue had no effect. siRNA experiments supported the involvement of PLC and knockdown of PLCβ3 but not PLCγ suppressed the BCG induced Ca^{2+} response.

3.3.3. The supernatant of BCG also stimulates Ca^{2+} singling

The BCG introduced into the bladder is a diverse solution containing the whole bacteria plus bacterial fragments from external cell wall, intracellular content, as well as smaller particles secreted from the bacteria itself. To look at smaller molecules/particles the supernatant of BCG (sBCG) (the supernatant from BCG centrifuged for 3 min at 14 000 rpm) was tested on bladder cancer cells. The Ca^{2+} response after sBCG stimulation resembled the one seen with BCG with the distinction of fewer oscillating cells. Pretreatment with PTX, U73122 and ET-18-OCH_3 following sBCG stimulation all suppressed the Ca^{2+} response.

3.3.4. BCG PLC-dependently affects cell proliferation

Uncontrolled proliferation is one of the hallmarks of cancer therefore BCG's effect on proliferation next was investigated using immunocytochemistry with Ki67 and EdU. Ki67 is present in nucleus of cells in all active cell cycle states (G1, S, G2 and M) but is absence in resting cells (G0), while EdU detects cells only in the s-phase of the cell cycle. These data demonstrated that treatment with BCG independently ofPLB-985 (DSMO-differentiated human promyelotic leukemia cells to mature neutrophil-like cells) decreased the amount of EdU positive cells but had no effect on Ki67 expression. BCG treated cells still seems to remain in the cell cycle but fewer cells reached the s-phase. However treatment with the PLC inhibitor U73122 reversed the BCG effect on EdU.

3.3.5. Conclusions

Here we showed that treatment of urinary bladder cancer (T24) cells with BCG induces an intracellular Ca^{2+} increase and reduce cell proliferation. Experiments with external Ca^{2+} free media gave a similar response as presence of extracellular Ca^{2+} indicating a Ca^{2+} release from internal stores. This was supported through depletion of the ER with cyclopiazonic acid (CPA) which blocked the BCG-induced Ca^{2+} response. Furthermore pharmacological block with U73122 or ET-18-OCH_3 or knockdown with small interference RNA for PLCβ3 repressed Ca^{2+} signaling and U73122 reversed the BCG induced effect seen on cell proliferation (Figure 9.). Inhibition with the G-protein receptor blocker pertussis toxin also blocked the observed effect. This finding suggests a role for PLC-dependent Ca^{2+} signaling in cell growth of urinary bladder cancer.

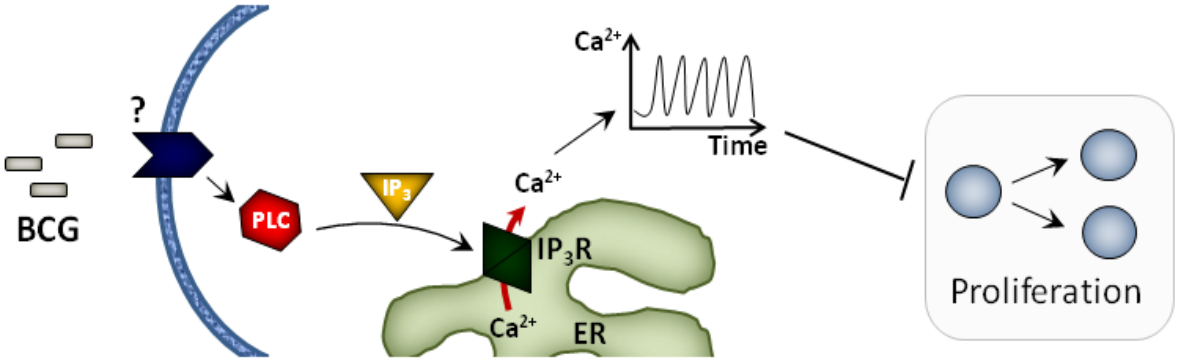

Figure 9. Schematic sketch over BCG signaling

4 General conclusions

This thesis presents three different studies showing three different roles for Ca^{2+} signaling of crucial importance to cell differentiation, cell proliferation, and cell death, three aspects highly coordinated with development and disease.

Paper I. We have demonstrated that ouabain, an endogenous steroid hormone, through binding to its receptor Na^+, K^+-ATPase can trigger dendritic outgrowth in cortical neurons. This occurs via a Ca^{2+} dependent transcriptional program including CaM kinases which regulates downstream CREB and CRE mediated gene expression. In addition this mechanism also includes Ca^{2+} oscillations and phosphorylation of MAP kinases. These data represents a new role for the Na^+, K^+-ATPase in dendritic growth.

Paper II. A novel role for the Ca^{2+} permeable HCN channel 2 as an upstream regulator of cell death has been shown. This is mediated through a prolonged Ca^{2+} elevation which leads to calpain activation and processing of AIF. AIF will be released from the mitochondria to the cytoplasm and translocates to the nucleus where is induces chromatin condensation.

Paper III. We showed that BCG triggers a PLC-dependent Ca^{2+} response in human bladder cancer cells that reduce cell proliferation and thereby cancer cell growth. Store depletion by SERCA inhibition blocked the BCG-triggered signal cascade suggesting a role of ER as a Ca^{2+} source. However, this signaling event was dependent on PLC since pharmacological inhibition or small interference RNA-mediated gene silencing abolished the response. Finally EdU incorporation showed that BCG-controlled cell proliferation was mediated via a Ca^{2+}- and PLC-dependent mechanism.

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Collaborators

Paper I: Luc Desfrere, Hiromi Hiyoshi, Seth Malmersjö, Evanthia Nanou, Manuel Estrada, Ayako Miyakawa, Hugo Lagercrantz, Abdeljabbar El Manira, Mark Lal

Paper II: Erik Norberg, Olga Korenovska, S Szydlowski, G Silberberg, Sten Orrenius, Boris Zhivotovsky.

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Mol Neuro

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Na,K-ATPase signal transduction triggers CREB activation and dendritic growth

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Dendritic growth is pivotal in the neurogenesis of cortical neurons. The sodium pump, or Na,K-ATPase, is an evolutionarily conserved protein that, in addition to its central role in establishing the electrochemical gradient, has recently been reported to function as a receptor and signaling mediator. Although a large body of evidence points toward a dual function for the Na,K-ATPase, few biological implications of this signaling pathway have been described. Here we report that Na,K-ATPase signal transduction triggers dendritic growth as well as a transcriptional program dependent on cAMP response element binding protein (CREB) and cAMP response element (CRE)-mediated gene expression, primarily regulated via Ca^{2+}/calmodulin-dependent protein (CaM) kinases. The signaling cascade mediating dendritic arbor growth also involves intracellular Ca^{2+} oscillations and sustained phosphorylation of mitogen-activated protein (MAP) kinases. Thus, our results suggest a novel role for the Na,K-ATPase as a modulator of dendritic growth in developing neurons.

Calcium signaling | CREB | Dendritic growth | Na,K-ATPase signaling | Neuronal development

Dendrites characterize neuronal cells from other cell types and allow neurons to form networks that are responsible for different functions in the brain. The growth of dendrites is a highly dynamic process that involves a series of orchestrated signaling events that take place during neurogenesis (1–3). In the developing cortex, several phenomena and signaling mechanisms have been demonstrated, including layer-specific dendritic growth and branching activated by neurotrophins (4), restricted dendritic growth via Notch1 signaling (5), and dendritic development via intracellular calcium (Ca^{2+}) signaling (6).

The sodium pump, or Na,K-ATPase, belongs to a family of evolutionarily ancient enzymes that catalyze active transport of cations through hydrolysis of adenosine triphosphate (ATP) across the cell membrane in all mammalian cells (7). In the central nervous system, mutations in Na,K-ATPase are known to cause neuronal dysfunction and neurodegeneration in *Drosophila* (8), familial hemiplegic migraine in humans (9), and rapid-onset dystonia-Parkinsonism (10). In addition to maintaining the electrochemical gradient, the Na,K-ATPase has been shown to act as a receptor and signal transducer. Previous reports indicate that Na,K-ATPase signal transduction stimulates proliferation in smooth muscle cells (11) and apoptosis in prostate cancer cells (12). Ouabain, an endogenously synthesized hormone (13, 14), is Na,K-ATPase's ligand and forms a hormone-receptor complex capable of inducing mitogen-activated protein (MAP) kinase phosphorylation (15, 16) and intracellular Ca^{2+} signaling (17–19).

In developing neurons, Ca^{2+} signaling has been shown to control proliferation, differentiation, and dendritic growth (6, 20–22) by acting on MAP kinases (23, 24) and Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (25, 26). Both of these kinases are known to control the transcription factor cAMP response element binding protein (CREB), which regulates cell proliferation and differentiation in a range of cell types in developing vertebrates (27–29). Because the CREB-activating pathways can also be triggered by Na,K-ATPase signaling, we investigated whether the Na,K-ATPase is involved in neuronal differentiation.

We report here that signal transduction activated by the Na,K-ATPase indeed modulates dendritogenesis in developing cortical neurons. This biological process involves a transcriptional program dependent on CREB-mediated and cAMP response element (CRE)-mediated gene expression via CaM kinases as well as MAP kinase phosphorylation and intracellular Ca^{2+} signaling. These data provide evidence for a novel signaling pathway by which the Na,K-ATPase stimulate dendritic growth in the developing brain and may thus play an important role in circuits wiring.

Results

Na,K-ATPase Receptor Activation Triggers Dendritic Growth in Cortical Neurons. The effect of Na,K-ATPase signal transduction on dendritic growth was studied in cortical neurons prepared from embryonic day (E) 18 rats. To visualize dendritic morphology of individual cells we immunolabeled cortical neurons with a MAP2 antibody at E18 + 4 days *in vitro* (DIV). This approach reveals the detailed dendritic morphology of cortical neurons in primary culture and can be applied to study the role of diverse signaling pathways in regulating dendritic growth (30). Neurons exposed to the steroid hormone ouabain (1 μmol/l) for 48 hours showed distinctive morphological changes observed as significantly enhanced dendritic growth (Figs. 1*A* and 1*B*). The effect of ouabain on Na,K-ATPase varies in a dose-dependent manner (31, 32); therefore, cortical neurons were exposed to graded concentrations of ouabain, and dendritic arbor growth was examined. As shown in Fig. 1*C*, both the length and number of dendrites significantly increased when cells were treated with ouabain concentrations of 0.5 μmol/l and 1 μmol/l. The dendritic lengths were extended almost 2-fold and the number of dendrites increased by ≈100% after 1 μmol/l ouabain for 48 hours compared with mock treatment. In contrast, ouabain concentrations of 10 μmol/l or higher did not enhance dendritic growth in cortical neurons (Fig. 1*C*). Consequently, the optimal concentration for dendritogenesis, 1 μmol/l ouabain, was used for all subsequent experiments.

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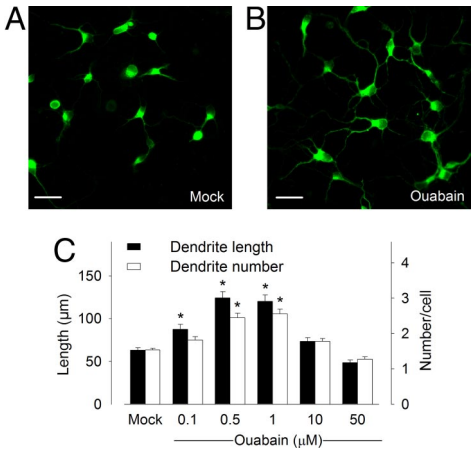

Fig. 1. Na,K-ATPase signal transduction triggers dendritic arbor growth in cortical neurons. (*A* and *B*) Confocal images of E18 cortical neurons in culture at 4 DIV immunolabeled with anti-MAP2 antibody treated with mock (*A*) versus 1 μmol/l ouabain (*B*) for 48 hours. Scale bars, 40 μm. (*C*) Quantification of length and arborization of MAP2-positive dendrites treated with various concentrations of ouabain, as indicated in the figure. Results are pooled from 10 randomly selected fields of view times three cultures. $* P < 0.05$ versus mock.

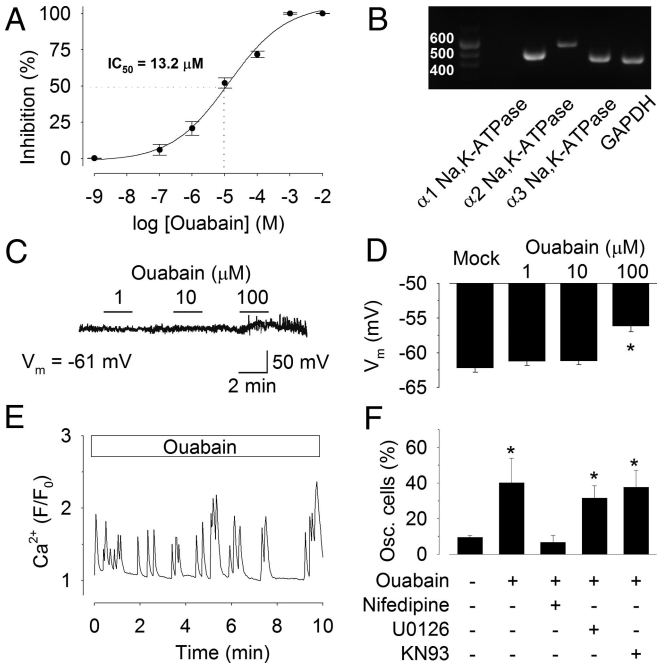

Fig. 2. Na,K-ATPase signaling occurs without changing the resting membrane potential. (*A*) Effect of ouabain on active $^{86}Rb^+$ transport by Na,K-ATPase. Cortical neurons at E18 + 4 DIV were incubated for 30 minutes with indicated concentrations of ouabain. The IC_{50} for the reduction in $^{86}Rb^+$ transport was 13.2 μmol/l ouabain ($n = 7$). (*B*) Expression of α-subunit Na,K-ATPase isoform-specific mRNA (cDNA) in E18 cortical neurons at 4 DIV. Amplification of the GAPDH-specific signal was performed as control. (*C*) Whole-cell patch-clamp recording of a cortical neuron at E18 + 4 DIV stimulated with 1, 10, or 100 μmol/l ouabain (resting membrane potential, -61 mV). (*D*) Quantitative data of the membrane potential in cortical neurons at E18 + 4 DIV stimulated with 1, 10, or 100 μmol/l ouabain. $* P < 0.05$ versus mock. (*E*) Representative single-cell Ca^{2+} recording of a Fluo-3/AM-loaded E18 cortical neuron in culture at 4 DIV treated with ouabain 1 μmol/l, 6 hours before initiating measurements. Ratio F/F_0 represents fluorescence intensity over baseline. (*F*) Quantification of the percentage of Na,K-ATPase signal-transduced Ca^{2+} oscillating cells after treatment with the inhibitors nifedipine, U0126, and KN93, respectively. $*P < 0.05$ versus mock.

Na,K-ATPase Signal Transduction Occurs Without Changing the Resting Membrane Potential. Ouabain is a well-established catalytic inhibitor of the Na,K-ATPase. To determine the extent to which ouabain inhibits Na,K-ATPase activity in cortical neurons, we carried out $^{86}Rb^+$-flux experiments. Uptake of $^{86}Rb^+$ is correlated with K^+ uptake and thus reflects the turnover rate of the pump. The results show that cortical neuronal Na,K-ATPase had an IC_{50} of 13.2 μmol/l (95% confidence interval) for ouabain and that the optimal concentration for dendritic growth, 1 μmol/l ouabain, inhibited active $^{86}Rb^+$ uptake only by 20.7 ± 4.8% ($n = 7$) (Fig. 2*A*). Previous studies have shown that Na,K-ATPase isoforms have different affinities for ouabain (31) as well as cell-type-specific and developmental-specific expression patterns (33). The α3- and α2-isoforms of Na,K-ATPase have a higher affinity to ouabain as compared with the rather insensitive α1-isoform (31, 32). Thus, we determined the identity of the α-subunit isoforms expressed in rat cortical neurons in primary culture by reverse transcription-polymerase chain reaction (RT-PCR) using sequence-specific primers. mRNA (cDNA) from three Na,K-ATPase α-subunit isoforms, including the neuron-specific α3-isoform, were detected in rat cortical neurons (Fig. 2*B*).

To rule out an effect of ouabain on the resting membrane potential, whole-cell patch-clamp recordings were made from cortical neurons. Ouabain at a concentration of 1 μmol/l or 10 μmol/l had no significant effect on the resting membrane potential of cortical neurons (Figs. 2*C* and 2*D*). At a higher concentration of 100 μmol/l, at which enzyme activity is inhibited by 72.2 ± 2.2% ($n = 7$), ouabain depolarized the membrane potential from -62.2 ± 0.7 mV to -56.1 ± 0.8 mV ($n = 12$) (Figs. 2*C* and 2*D*). These results demonstrate that ouabain does not alter the resting membrane potential at concentrations that promote dendritogenesis.

Calcium signaling exerts a profound influence on dendritic growth during development (6). We therefore examined the effect of Na,K-ATPase signal transduction on Ca^{2+} signaling in cortical neurons. Intracellular Ca^{2+} dynamics were imaged in neuronal cells loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. When cortical neurons were exposed to 1 μmol/l ouabain for 6 hours, the treatment that elicited a robust effect on dendritic growth, slow intracellular Ca^{2+} oscillations were induced (Fig. 2*E*). Intracellular Ca^{2+} signaling was also observed in untreated cells, 9.5 ± 0.9% ($n = 147$) but was significantly increased in neurons treated with ouabain, 40.1 ± 13.8% ($n = 129$) (Fig. 2*F*). These Ca^{2+} oscillations were strongly suppressed by the L-type Ca^{2+} channel blocker nifedipine, 6.7 ± 4.0% ($n = 140$) (Fig. 2*F* and [supporting information (SI) Fig. S1*A*]). Neither inhibition of MAP kinases by U0126 nor CaM kinases by KN93 abolished ouabain-induced Ca^{2+} oscillations in cortical neurons (below and Fig. 2*F*).

MAP Kinase Phosphorylation Is Stimulated by Na,K-ATPase Signaling. Activation of MAP kinases plays an important role, largely through regulation of gene transcription, in a number of cellular processes including cell proliferation, DNA synthesis, differentiation, and survival (23, 34, 35). In neurons, MAP kinases have been shown to regulate dendritic growth and morphology (24, 36). To assess whether Na,K-ATPase signal transduction activates p42/44 MAP kinase (referred to as ERK1/2) in cortical neurons, we exposed neurons to ouabain and monitored ERK1/2 phosphorylation using Western blotting. When cells were examined after 10 minutes of ouabain treatment no ERK1/2 phosphorylation was observed (data not shown). Cortical neurons exposed to ouabain for 4 hours and 6 hours, however, showed a sustained and significant ERK1/2 phosphorylation, which was abolished using the selective MEK inhibitor U0126 (Fig. 3*A*). Sustained activation of MAP kinases has previously been asso-

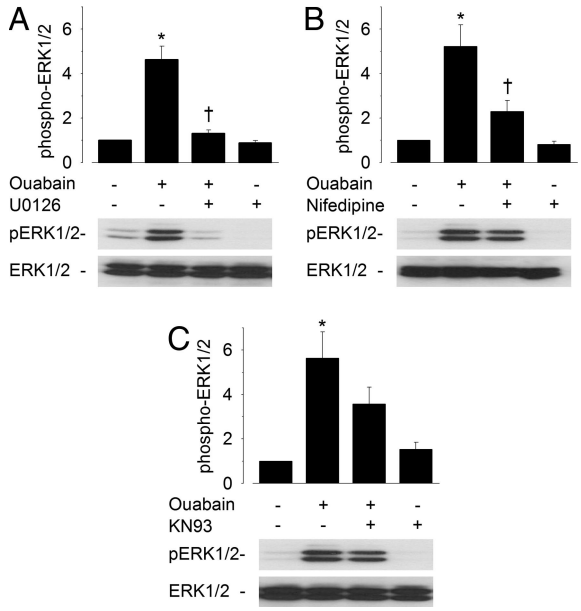

Fig. 3. MAP kinase activation is triggered by Na,K-ATPase signal transduction. (*A-C*) Western blot of Na,K-ATPase signal-transduced phosphorylated 42/44 MAP kinase (pERK1/2) in cortical neurons at E18 + 4 DIV treated with 1 μmol/l ouabain for 6 hours plus MAP kinase inhibitor U0126 (*A*), L-type Ca^{2+} channel blocker nifedipine (*B*), and CaM kinase inhibitor KN93 (*C*). Each bar represents the fold increase of phospho-ERK1/2 relative to mock after normalizing against total nonphosphorylated ERK1/2. $*P < 0.05$ versus mock; $^{\dagger}P < 0.05$ versus ouabain-treated cells.

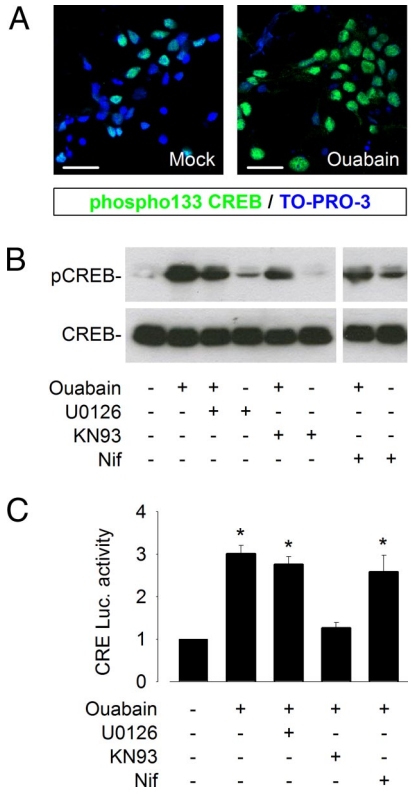

Fig. 4. CREB activation induced by Na,K-ATPase signal transduction is mediated via CaM kinases. (*A*) Confocal images of phospho-Ser-133 CREB-immunostained (green) E18 cortical neurons in culture at 5 DIV treated with mock (*left panel*) and with ouabain 1 μmol/l (*right panel*) for 6 hours. Nuclear staining with TO-PRO3 (blue). (*B*) Levels of CREB phosphorylation as detected by phospho-Ser-133 CREB Western blot of cortical neurons at E18 + 5 DIV treated with 1 μmol/l ouabain for 6 hours, preincubated with MAP kinase inhibitor U0126, CaM kinase inhibitor KN93, and L-type Ca^{2+} channel blocker nifedipine (Nif), respectively. (*C*) CREB-mediated transcription, measured using a CRE-luciferase reporter gene transfected into E18 cortical neurons in culture at 3 DIV, of Na,K-ATPase signal transduction in cortical neurons at E18 + 5 DIV treated with mock versus 1 μmol/l ouabain for 6 hours, preincubated with MAP kinase inhibitor U0126, CaM kinase inhibitor KN93, and L-type Ca^{2+} channel blocker nifedipine (Nif), respectively. Each bar represents the fold increase relative to mock after normalizing against Renilla. $*P < 0.05$ versus mock.

ciated with physiological phenomena such as gene transcription and dendritic growth (34, 37). We then tested whether MEK and MAP kinases had any effect on the Ca^{2+} oscillatory response evoked by treating cells with ouabain for 6 hours. U0126 did not block ouabain-induced Ca^{2+} oscillations (Fig. S1*B*) and had no significant effect on the number of responding cells, 31.6 ± 6.9% (n = 118), when compared with ouabain treatment alone (Fig. 2*F*). To determine the effect of Ca^{2+} signaling induced by Na,K-ATPase signal transduction on ERK1/2 activation, we next examined cells preincubated with the Ca^{2+} signaling inhibitor nifedipine. Cortical neurons exhibited a reduced level of Na,K-ATPase signal transduced ERK1/2 phosphorylation after nifedipine pretreatment (Fig. 3*B*).

The family of CaM kinases is a principal downstream target of Ca^{2+} signaling that is known to regulate neuronal differentiation and dendritic growth (25, 26, 29). To test whether these kinases were involved in mediating Na,K-ATPase signal transduction in cortical neurons, cells were stimulated with ouabain in the absence or presence of the general CaM kinase inhibitor KN93. After this treatment, phosphorylation of ERK1/2 was partially, yet not significantly (P = 0.3, one-way analysis of variance [ANOVA]), inhibited in cortical neurons (Fig. 3*C*). Next we investigated whether CaM kinases were involved in the ouabain-induced Ca^{2+} oscillations. Pretreatment with KN93 did not abolish Na,K-ATPase signal transduced Ca^{2+} oscillations, 37.7 ± 9.6% (n = 134) (Fig. 2*F* and Fig. S1*C*). These results indicate that activation of MAP kinases by Na,K-ATPase signal transduction involves the synergistic actions of MEK, Ca^{2+} oscillations, and CaM kinases.

Na,K-ATPase Signal Transduction Triggers CREB Transcription Through CaM Kinases. The transcription factor CREB is implicated in neuronal differentiation (26, 27), where it is strongly regulated via CaM kinases (28, 38). To determine the effect of Na,K-ATPase signal transduction on CREB activation we first carried out immunocytochemistry experiments using an antibody specific for Ser-133-phosphorylated CREB. After ouabain treatment for 6 hours, immunostaining revealed a marked increase of phosphorylated CREB within the nucleus (Fig. 4*A*). The nuclear localization of CREB was confirmed by co-labeling cortical neurons with the nuclear stain TO-PRO3 (Fig. 4*A*). The role of Na,K-ATPase signal transduction on CREB activation was further investigated with Western blot experiments using the Ser-133 phospho-CREB antibody. These experiments showed that CREB was strongly Ser-133 phosphorylated by Na,K-ATPase signal transduction (Fig. 4*B*).

Next we examined which signaling cascades were involved in the Na,K-ATPase signal-transduced CREB Ser-133 phosphorylation. Inhibiting MAP kinases using U0126 reduced the immuno signal detected by the Ser-133 phospho-CREB antibody (Fig. 4*B*). CREB Ser-133 phosphorylation was also downregulated by the CaM kinase blocker KN93 (Fig. 4*B*). CaM kinases are activated by Ca^{2+} signaling; consequently, when Ca^{2+} oscillations were blocked with nifedipine, a reduction in the CREB Ser-133 phosphorylation was observed (Fig. 4*B*). This set of experiments suggests that each of these pathways is partially involved in CREB Ser-133 phosphorylation via Na,K-ATPase

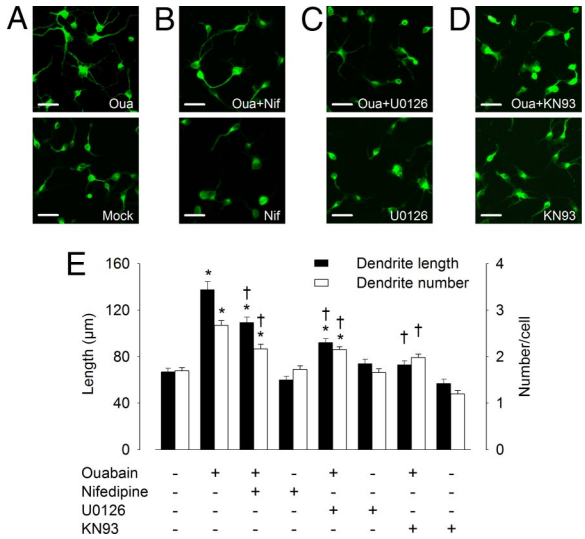

Fig. 5. Dendritic growth in cortical neurons induced by Na,K-ATPase signal transduction involves crosstalk between Ca^{2+} signaling, MAP kinases, and CaM kinases. (*A-D*) Confocal images of E18 cortical neurons at 4 DIV immunolabeled with anti-MAP2 antibody treated with 1 μmol/l ouabain (A) together with L-type Ca^{2+} channel inhibitor nifedipine (Nif) (*B*), MAP kinase inhibitor U0126 (*C*), and CaM kinase inhibitor KN93 (*D*) for 48 hours. Scale bars, 40 μm. (*E*) Quantification of length and arborization of MAP2-positive dendrites treated with 1 μmol/l ouabain together with nifedipine, U0126, and KN93, respectively. Results are pooled from 10 randomly selected fields of view times three cultures. $*P < 0.05$ versus mock; $^{\dagger}P < 0.05$ versus ouabain-treated cells.

signal transduction. Although phosphorylation of CREB at Ser-133 is required for inducing CREB-dependent transcription, there are instances in which Ser-133 phosphorylation is not sufficient for target gene activation (28). To further investigate CREB activation induced by Na,K-ATPase signal transduction, cortical neurons cells were transfected with a CRE-luciferase reporter gene that is activated by endogenous CREB. A significant 3-fold increase in CRE-luciferase activity was observed when cells were exposed to ouabain for 6 hours (Fig. 4*C*). Whereas U0126 and nifedipine resulted in only a marginal reduction in ouabain-induced CRE-luciferase activity, KN93 completely abolished the CREB activation triggered by Na,K-ATPase signal transduction (Fig. 4*C*). Taken together these results suggest that CREB activation in cortical neurons triggered by Na,K-ATPase signal transduction predominantly occurs through a CaM kinase-dependent mechanism.

Dendritic Growth Induced by Na,K-ATPase Signaling Is Orchestrated by MAP Kinases, CaM Kinases, and Extracellular Ca^{2+} Influx. Finally, we examined the impact of Ca^{2+} signaling, MAP kinases, and CaM kinases on Na,K-ATPase signal-transduced dendritic growth in cortical neurons. A partial decrease in ouabain-triggered dendritic length and arborization was observed in response to nifedipine treatment, suggesting that L-type channel-mediated Ca^{2+} influx plays a role in dendritic growth (Fig. 5*B*). Inhibition of MAP kinase signaling with U0126 significantly attenuated dendritogenesis in ouabain-treated cells (Fig. 5*C*). Although nifedipine and U0126 both significantly decreased ouabain-induced neurite outgrowth, neither inhibitor was able to completely abrogate the Na,K-ATPase signal-transduced effects on dendritic growth and arborization in cortical neurons. Inhibition of CaM kinases with KN93, however, resulted in complete inhibition of ouabain-induced dendritic branching (Fig. 5*D*). These observations support a model in which Na,K-ATPase signal transduction triggers dendritic growth via a signaling pathway that involves extracellular Ca^{2+} influx as well as MAP kinase and CaM kinase-modulated CREB transcription.

Discussion

The findings presented in this report indicate that Na,K-ATPase signal transduction induces dendritogenesis in cortical neurons via activation of a transcriptional program that involves CREB- and CRE-mediated gene expression, primarily through a CaM kinase-dependent signaling pathway. This signaling event is induced by exposing cells to a concentration of ouabain that does not affect the resting membrane potential. Persistent Ca^{2+} oscillations and sustained MAP kinase phosphorylation also participate in this signaling cascade, as blocking L-type Ca^{2+} channels or MEK suppresses downstream effects.

Ouabain is an endogenous steroid hormone that is present in mammalian tissues; it has been isolated from hypothalamus (39), adrenals (40), and human plasma (41). Evidence indicates that circulating levels of ouabain and ouabain-like factors are elevated during pregnancy and in newborn infants (13, 32), thereby suggesting a developmental role for this signaling molecule. Our results, which show that Na,K-ATPase, the receptor of ouabain, stimulates dendritic growth in rat cortical neurons, support such a biological function for ouabain. Concentrations of endogenous ouabain in the embryonic human brain during the course of development are unknown but are predicted to lie in the subnanomolar-to-nanomolar range, as previously reported (13, 32). Indeed, nanomolar concentrations of ouabain stimulates neurite outgrowth in human neuroblastoma cells (Fig. S2). Furthermore, in the brain, there are multiple Na,K-ATPase isoforms that each have cell-type-specific and developmental-specific expression patterns (33, 42) as well as different ouabain affinities (31, 42). These spatial and temporal expression patterns of Na,K-ATPase isoform remain largely unknown but may be involved in the development and wiring of the brain. As such, the extent to which the different α-subunit isoforms contribute to the overall cellular effects of ouabain remain to be elucidated.

The mechanism by which Na,K-ATPase triggers signal transduction remains disputed, with two principal hypotheses being proposed. One mechanism suggests that partial inhibition of the Na,K-ATPase leads to an increased intracellular Na^+ concentration that transactivates Na^+/Ca^{2+}-exchangers, which thereby produce cytosolic Ca^{2+} waves that are sufficient to elicit downstream signaling events (18, 43). The other mechanism suggests that inhibition of Na,K-ATPase pumping activity is not in itself required for a signaling event to occur; instead, Na,K-ATPase is proposed to function via a microdomain where ouabain-induced signaling occurs via physical association of Na,K-ATPase with a host of binding partners. Na,K-ATPase has been reported to associate with the inositol 1,4,5-trisphosphate receptor to generate intracellular Ca^{2+} oscillations (44) and to form a complex with the epidermal growth factor receptor, leading to MAP kinase activation (16). Dendritic growth via Na,K-ATPase signal transduction might be modulated by either or both of these signaling pathways.

Using electrophysiological recordings it was shown that 1 μmol/l ouabain, the optimal concentration for inducing dendritogenesis in cortical neurons, apparently does not change the resting membrane potential. Ouabain at 100 μmol/l, a concentration that did not induce dendritic growth, however, did depolarize the membrane potential. Thus, ouabain-induced depolarization alone does not appear to be sufficient to trigger dendritic growth in cortical neurons. These data, together with the $^{86}Rb^+$-flux experiments, suggest that inhibition of Na,K-ATPase pumping activity *per se* is not the mechanism responsible for triggering this biological process. The observation that Na,K-ATPase signal transduction is not a fast signaling event is further strengthened by the experiments on Ca^{2+} signaling and MAP kinases, which both show delayed responses. Persistent

Ca^{2+} oscillations and sustained ERK1/2 phosphorylation were detected after 6 hours of pretreatment with 1 μmol/l ouabain. Delayed and sustained phosphorylation of MAP kinases has previously been implicated in neuronal differentiation (45). These observations indicate that the signaling mechanism by which dendritic growth is induced in cortical neurons does not depend on changes in the resting membrane potential but, rather, involves a slow process that takes hours to activate.

Analysis of Ser-133 phosphorylation of CREB- and CRE-mediated gene expression in cortical neurons treated with ouabain gave rise to somewhat disparate results. This might be caused by the many phosphorylation sites on the CREB protein that differentially regulate total CREB activity (27, 28, 46). For example, phosphorylation at Ser-133 increases CREB activity, whereas Ser-142 phosphorylation inhibits CREB (38). Therefore we cannot directly correlate Ser-133 phosphorylation of CREB with CRE-dependent transcription. Phosphorylated CREB is also known to bind the Ca^{2+}-dependent CREB binding protein (CBP) to form a molecular complex that determines CRE transcriptional activity (47). The differential ability of CaM kinases and MAP kinases to activate CBP (48) may be the molecular distinction that underlies the distinct biological effects of these kinases. This difference may explain why inhibiting MAP kinases decreased Ser-133 phosphorylation of CREB but failed to block CRE activation induced by Na,K-ATPase signaling. Furthermore, CREB activation was previously demonstrated to occur via an interplay between MAP kinases and CaM kinases, in which MAP kinases were involved in a more sustained phosphorylation of CREB whereas the fast onset of CREB phosphorylation was triggered by CaM kinases (34, 46, 49). Taken together, our results suggest that Na,K-ATPase receptor activation leads to CREB activation and dendritic growth via crosstalk between the MAP kinase and CaM kinase pathways.

Our findings suggest that Na,K-ATPase signal transduction stimulates dendritic growth in cortical neurons via activation of a transcriptional program that involves CREB- and CRE-mediated gene expression. The signaling pathway is primarily regulated via CaM kinases, but crosstalk with MAP kinase phosphorylation and Ca^{2+} oscillations also participates in this event. This type of mechanism may provide a novel basis for neuronal differentiation through Na,K-ATPase signal transduction and induction of a specific program of gene expression that is critical for the developing nervous system.

Materials and Methods

Cell Cultures. Cerebral cortical neurons in primary culture were prepared from Sprague-Dawley rat fetuses at embryonic day (E)18. The cerebral cortices were dissected into Hank's balanced salt solution (HBSS). HBSS was removed and tissues were treated with 0.25% trypsin at 37 °C for 15 minutes. Tissues were then treated with 0.1% DNase I at 37 °C for 5 minutes. Cells were diluted to 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mmol/l L-glutamine, and were seeded on plates precoated with 40 μg/ml poly-L-lysine. After 3 hours, DMEM was replaced with neurobasal medium (GIBCO) containing B27 Supplement (GIBCO), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mmol/l L-glutamine. Half of the cell culture medium was replaced after 3 DIV with neurobasal medium containing the supplements specified above and aracytine 10^{-7} mol/l.

Reagents. Reagents and concentrations, unless otherwise stated, were as follows,: ouabain (1, 10, 100 μmol/l, Sigma), nifedipine (50 μmol/l, Sigma), KN93 (20 μmol/l, Tocris), and U0126 (20 μmol/l, Cell Signaling).

$^{86}Rb^+$ Uptake Assay. Cortical neurons at E18 + 4 DIV were rinsed with phosphate buffered saline solution (PBS) and incubated with PBS containing indicated ouabain concentrations for 30 minutes at 37 °C. To each well $^{86}Rb^+$ was added at $\approx$1.5 μCi/ml, and incubation was continued for another 10 minutes in 37 °C. Uptake was thereafter inhibited by 2 mmol/l ouabain, and this value was taken as the maximal rate of active uptake. The incubation was stopped by rinsing the plate four times with PBS plus $BaCl_2$ (5 mmol/l). Cells were extracted with 0.3 ml of 1 mol/l NaOH for 10 minutes, and samples were counted in a scintillation counter. Each data point represents the average of the radioactivity present in four separate wells.

RT-PCR. Total RNA was extracted from cultured rat embryonic cortical neurons at E18 + 4 DIV using TRIzol reagent (Invitrogen). Single-strand cDNA was synthesized from 1 μg total RNA by random primers and SSII-RT (Invitrogen) and the cDNA was then amplified by RT-PCR with TaqDNA polymerase (Invitrogen). The PCR reactions were performed as follows: 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 60 seconds, repeated for 45 cycles. The PCR products were separated on a 1.5% agarose gel, and ethidium bromide was used for visualization.

Calcium Imaging. Calcium experiments on cortical neurons at E18 + 4 DIV were carried out in artificial cerebrospinal fluid containing the following: 125 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 1.25 mmol/l NaH_2PO_4, 5 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, and 20 mmol/l D-glucose bubbled with 95% O_2 and 5% CO_2 to reach a final pH of 7.3. Cells were incubated for 15–30 minutes at 37 °C with 5% CO_2 in 5 μmol/l Fluo-3/AM (Molecular Probes, Invitrogen). Coverslips were mounted in a temperature controlled (37 °C) chamber (Warner Instruments) and clamped onto a Zeiss Axiovert 100M microscope, equipped with a C-Apochromat 40X/1.2NA water immersion objective (Zeiss), connected to a Lambda LS xenon-arc lamp (Sutter), Lambda 10–3 filter-wheel (Sutter), and a *smart*Shutter (Sutter). Images were acquired at 0.2 Hz with an EMCCD camera Cascade II:512 (Photometrics) controlled by the acquisition software MetaFluor (Molecular Devices). Perfusion (1 ml/min) with 95% O_2-5% CO_2 bubbled artificial cerebrospinal fluid was used for Ca^{2+} recordings on cortical neurons.

Electrophysiology. Cortical neurons in culture at E18 + 4 DIV were recorded in the whole-cell configuration using a patch-clamp amplifier (AxoPatch 200A, Axon Instruments). Neurons had an average resting membrane potential of -62.2 ± 0.7 mV and were continuously perfused with extracellular solution containing the following: 100 mmol/l NaCl, 2 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 10 mmol/l glucose, and 10 mmol/l HEPES, with pH adjusted to 7.6 using NaOH, and 223 mOsm. The pipettes were filled with a solution containing: 90 mmol/l K-gluconate, 5 mmol/l KCl, 0.1 μmol/l $CaCl_2$, 10 mmol/l HEPES, 4 mmol/l Mg_2ATP, 0.3 mmol/l Na_4GTP, and 5 mmol/l phosphocreatine Na_2 with pH adjusted to 7.4 using KOH, and 217 mOsm. Solutions and drugs were applied through a gravity-driven microperfusion system, with the tip placed close to the recorded cell.

Western Blot and Immunocytochemistry. Cortical neurons at E18 + 4 DIV were exposed to various treatments. The cells were lysed using modified RIPA buffer for 20 min at 4 °C. Protein concentration was determined using a BCA protein assay (Pierce) and equal amounts of cellular protein ($\approx$10–20 μg) were separated on a 10% sodium dodecyl sulfate gel electrophoresis, followed by a transfer to a nitrocellulose membrane. Membranes were blocked in 5% skim milk in Tris-buffered saline solution plus 0.5% Tween-20 for 1 hour before incubation with primary antibodies (Ab) (1:1000) (ERK1/2, Phospho-ERK1/2, Ser-133 CREB, and Phospho-Ser-133 CREB, all from Cell Signaling) overnight at 4 °C and further incubation with horseradish peroxidase-conjugated secondary Ab (1:5000) (Amersham) for 1 hour. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Amersham).

Immunocytochemical staining of phospho-Ser-133 CREB and MAP2 was performed according to standard protocol, using fixation by 4% paraformaldehyde in 1 hour. After blocking with 1% bovine serum albumen, cells were incubated with phospho-Ser-133 CREB Ab (1:100, Cell Signaling) or MAP2 Ab (1:400, Chemicon) overnight and then with Alexa 488 secondary Ab (1:2000, 1:200; Molecular Probes) for 1 hour, together with 0.3% Triton X-100. Nucleus was stained with TO-PRO-3 (1:200; Molecular Probes) for 10 minutes. Slides were mounted using the Prolong Antifade Kit (Molecular Probes) and scanned in a Zeiss LSM510 confocal microscope equipped with a C-Apochromat 40X/1.2 water immersion objective (Zeiss).

Luciferase Assay. For CREB activity assay, cortical neurons were transfected at E18 + 3 DIV with pCRE-luciferase reporter (Stratagene) and pRL-TK Renilla reporter (Promega) using Lipofectamine 2000 (Invitrogen). After 48 hours, cells were treated with KN93, U0126, or nifedipine for 15 minutes followed by ouabain for 6 hours. Thereafter cells were harvested, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

Data Analysis. Dendritic growth was analyzed from 10 fields of view per cell culture using ImageJ (National Institutes of Health, Bethesda, MD) together with the plug-in NeuronJ. For each field, the average dendritic

length and/or the number of dendrites per cell was calculated. When recording, intracellular Ca^{2+}, cells were considered oscillating when at least three Ca^{2+} transients were observed exceeding 50% of the baseline. Data are presented as mean ± SE of a minimum of three experiments unless otherwise indicated. Statistical significance was accepted at $P < 0.05$ as determined by analysis of variance, followed by Bonferroni's *post hoc* test for multiple comparisons.

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Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis

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Cellular calcium uptake is a controlled physiological process mediated by multiple ion channels. The exposure of cells to either one of the protein kinase C (PKC) inhibitors, staurosporine (STS) or PKC412, can trigger Ca^{2+} influx leading to cell death. The precise molecular mechanisms regulating these events remain elusive. In this study, we report that the PKC inhibitors induce a prolonged Ca^{2+} import through hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) in lung carcinoma cells and in primary culture of cortical neurons, sufficient to trigger apoptosis-inducing factor (AIF)-mediated apoptosis. Downregulation of HCN2 prevented the drug-induced Ca^{2+} increase and subsequent apoptosis. Importantly, the PKC inhibitors did not cause Ca^{2+} entry into HEK293 cells, which do not express the HCN channels. However, introduction of HCN2 sensitized them to STS/PKC412-induced apoptosis. Mutagenesis of putative PKC phosphorylation sites within the C-terminal domain of HCN2 revealed that dephosphorylation of Thr^{549} was critical for the prolonged Ca^{2+} entry required for AIF-mediated apoptosis. Our findings demonstrate a novel role for the HCN2 channel by providing evidence that it can act as an upstream regulator of cell death triggered by PKC inhibitors.
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Introduction

Ca^{2+}-mediated signal transduction regulates multiple cellular processes, including cell motility, DNA transcription, exocytosis and cell death (Berridge *et al*, 1998). The physiological functions are normally governed by Ca^{2+} oscillations, whereas perturbation of the intracellular Ca^{2+} homeostasis may lead to cell death by either apoptosis or necrosis. The cytotoxic Ca^{2+} response is generally more pronounced and sustained than physiological Ca^{2+} signalling and can be due to excessive Ca^{2+} influx by plasma membrane ion channels, or to inappropriate Ca^{2+} release from intracellular stores, notably, the endoplasmic reticulum (Orrenius *et al*, 2003).

Non-small-cell lung carcinomas (NSCLC) are resistant to conventional radio- and chemotherapy. Nevertheless, NSCLC cells were shown to be sensitive to staurosporine (STS) and to the more selective protein kinase C (PKC) inhibitor, PKC412 (*N*-benzoyl-staurosporine), both of which trigger apoptosis in these cells (Joseph *et al*, 2002; Gallego *et al*, 2004). Recently, we reported that STS-induced cell death signalling in NSCLC cells involved both a stimulation of Ca^{2+} influx into the cells and an increase in the intracellular level of reactive oxygen species (ROS); both these events were required for the mitochondrial processing and release of apoptosis-inducing factor (AIF) and subsequent apoptosis (Norberg *et al*, 2008, 2010a, b).

AIF is a 62-kDa flavoprotein with NADH oxidase activity that is anchored to the inner mitochondrial membrane. The flavine adenine dinucleotide domain is essential for the redox function of AIF, but it does not contribute to its apoptotic function. To promote apoptosis, AIF must first be liberated from its membrane anchor, generating a soluble 57-kDa AIF fragment, that can be released from the mitochondria and translocate to the nucleus, where it promotes chromatin condensation and large-scale DNA fragmentation (Otera *et al*, 2005; Hangen *et al*, 2010) AIF-mediated apoptosis is independent of caspase activation and represents an important cell death mechanism in neurons and cells of neuroendocrine origin, for example, NSCLC (Norberg *et al*, 2010b).

So far, it has been unclear how the PKC inhibitors trigger Ca^{2+} influx into sensitive cells, and through which plasma membrane ion channel(s) Ca^{2+} enters the cell. Potential candidates are the family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. There are four HCN channels (HCN1–4) described, which are permeable to Na^+ and K^+ (ratio 1:4) and can be inhibited by mM concentrations of Cs^+ (Craven and Zagotta, 2006; Biel *et al*, 2009). Interestingly, two of the four channels, namely, HCN2 and HCN4, were demonstrated to also be permeable to Ca^{2+}, in addition to Na^+ and K^+ (Yu *et al*, 2004; Michels *et al*, 2008). However, the physiological implications of Ca^{2+} signalling through these channels are not known.

To investigate whether the HCN channels might participate in apoptosis signalling, we used NSCLC cells and primary

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cultures of rat cortical neurons exposed to PKC412 or STS as experimental model systems. Our data suggest that both kinase inhibitors caused Ca^{2+} influx through the HCN channel 2 by dephosphorylation of Thr^{549} within the internal regulatory domain of HCN2. The imported Ca^{2+} triggered apoptosis mediated by the calpain–AIF axis.

Results

PKC inhibitors trigger hyperpolarization-induced Ca^{2+} entry into NSCLC cells

We have previously reported that PKC inhibitors can trigger a Ca^{2+} influx from the extracellular medium into NSCLC cells (Norberg *et al*, 2008). To examine which plasma membrane Ca^{2+} channels might be involved in this response, we first tested the effects of known inhibitors of the voltage-dependent Ca^{2+} channels (VDCCs), such as nifedipine, Ni^{2+}, Ba^{2+} and La^{3+}. None of these inhibitors could prevent STS/PKC412-induced Ca^{2+} influx into the cells (Supplementary Figure S1). The majority of the VDCCs are activated by depolarization, and only few examples of hyperpolarization-activated Ca^{2+} channels exist (Siegelbaum, 2000). To analyse if STS or PKC412 might affect the polarization of the plasma membrane, cells were loaded with a polarization-sensitive dye, DiBAC, and analysed by time-lapse single-cell recordings (Figure 1A) as well as by flow cytometry (Figure 1B) Hyperpolarization was confirmed by patch-clamp recordings (Supplementary Figure S2). Thus, an early hyperpolarization of the cells was detected upon exposure to any one of the two PKC inhibitors.

Next, the effects of STS/PKC412 on the intracellular Ca^{2+} level in U1810 and H661 NSCLC cell lines were monitored. Figure 1C depicts representative single-cell Ca^{2+} responses in cells loaded with the Ca^{2+}-sensitive dye Fluo-4/AM and exposed to either one of the kinase inhibitors. Both PKC412 and STS induced a prolonged elevation of the intracellular Ca^{2+} level in the two cell lines. Yet, other kinase inhibitors, such as Ro-31-8220 and Wortmannin, did not stimulate Ca^{2+} entry into the cells (Supplementary Figure S3).

As mentioned above, only a few ion channels can be activated by hyperpolarization. One example is the family of HCN channels. Interestingly, two of these channels (HCN2 and HCN4) were demonstrated to also be permeable to Ca^{2+}, in

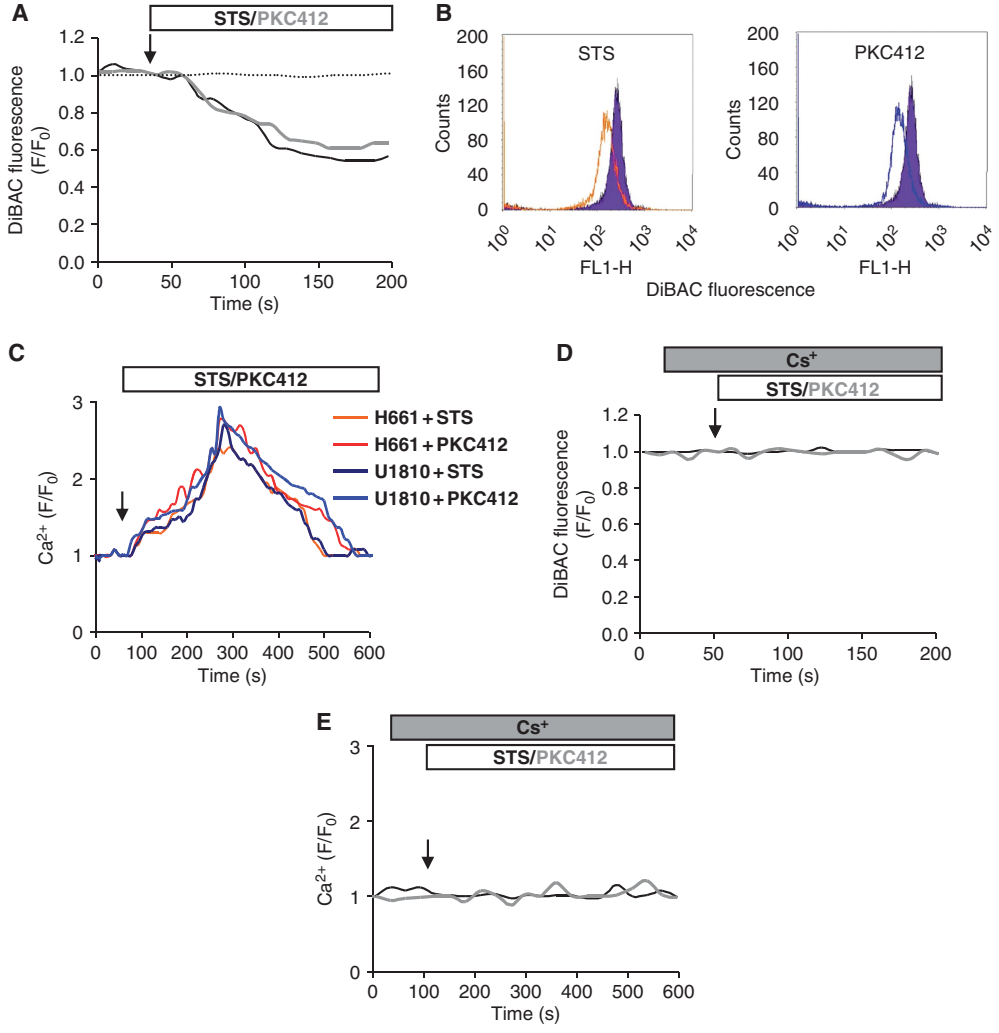

Figure 1 Protein kinase C inhibitors trigger hyperpolarization-mediated Ca^{2+} entry into NSCLC cells. U1810 cells were loaded with DiBAC, exposed to 0.2–1 μM STS or 1–5 μM PKC412 and analysed for the polarization of the plasma membrane using (**A**) confocal microscopy or (**B**) flow cytometry. (**C**) Representative single-cell Ca^{2+} recordings of Fluo-4/AM-loaded cells exposed to 0.2–1 μM STS or 1–5μM PKC412. (**D**) DiBAC-loaded cells were exposed to 3 mM CsCl before STS or PKC412 treatment. (**E**) Fluo-4/AM-loaded cells were pre-exposed to 3 mM CsCl before STS or PKC412 treatment. Ratio F/F_0 represents fluorescence intensity over baseline.

addition to Na^+ and K^+ (Yu *et al*, 2004; Michels *et al*, 2008). However, the cellular implications of Ca^{2+} signalling by these channels are not known. Therefore, the possible contribution of HCN channels to the Ca^{2+} entry into cells exposed to PKC inhibitors was investigated. First, the two NSCLC cell lines were exposed to Cs^+ before treatment with STS or PKC412, then plasma membrane polarization (Figure 1D) and the cytosolic Ca^{2+} level (Figure 1E) were monitored. Preincubation with Cs^+ inhibited both the hyperpolarization and the Ca^{2+} entry into STS/PKC412-treated cells, suggesting that under these conditions hyperpolarization was required for the Ca^{2+} influx, and that this occurred through the HCN channel(s).

PKC inhibitor-induced Ca^{2+} influx through the HCN2 channel leads to cell death

To investigate whether one of the Ca^{2+}-permeable HCN channels was involved in the Ca^{2+} import triggered by STS/PKC412, the mRNA and protein expression of HCN2/HCN4 was analysed by RT–PCR, immunocytochemistry and FACS, respectively. Both HCN2 and HCN4 mRNAs were expressed in U1810 (Figure 2A) and H661 cells (Supplementary Figure S4). However, the protein expression was detected only for the HCN2 channel, and the immunostainings confirmed its typical plasma membrane localization (Figure 2A).

To examine the possible role of HCN channels in the PKC inhibitor-induced Ca^{2+} entry, a siRNA approach was chosen. The levels of mRNA (HCN2/HCN4) and protein (HCN2) expression after siRNA downregulation are shown in Figure 2B (left and right panel, respectively). Next, cells were loaded with Fluo-4/AM and exposed to either one of the two PKC inhibitors. Representative Ca^{2+} traces, shown in Figure 2C, demonstrate that HCN2, but not HCN4, depletion abolished the kinase inhibitor-induced Ca^{2+} entry. To confirm this, Fluo-4/AM-loaded cells were treated with STS/PKC412 (as in Figure 2C), and 10 000 cells were analysed by flow cytometry (Figure 2D). In order to exclude off-target effect of the siRNA, two distinct non-overlapping siRNAs specific for HCN2 were used. Obtained results confirmed that the HCN2 channel indeed is mediating the influx of Ca^{2+} (Supplementary Figure S9A).

In addition, a rescue experiment was performed, in which HCN2 was first downregulated by siRNA in U1810 cells, and then mHCN2 was introduced. Ca^{2+} recordings revealed that the original phenotype observed after treatment with STS was restored by introducing mHCN2 (Supplementary Figure S5). Furthermore, to investigate whether Ca^{2+} influx by the HCN2 channel was sufficient to trigger apoptosis, the siRNA approach was used before exposure of the cells to STS/PKC412, and the number of cells with condensed nuclei was counted. Although chromatin condensation was observed in many of the control cells treated with STS (Figure 2E, left panel), downregulation of HCN2 significantly delayed this type of cell death manifestation (Figure 2E, right panel). Altogether, our results indicate that the STS/PKC412-induced Ca^{2+} influx by the HCN2 channel was sufficient to trigger an apoptotic response in NSCLC cells.

Ca^{2+} entry through HCN2 channels triggers caspase-independent, AIF-mediated cell death

To examine the mechanism of cell death that was triggered by the Ca^{2+} influx through the HCN2 channel, we first analysed if calpain was activated by monitoring the cleavage of two selective calpain substrates, Atg5 (Figure 3A) (Yousefi *et al*, 2006) and AIF (Figure 3B), in control cells and cells depleted of HCN2 channels. As shown in Figure 3A, STS-stimulated Atg5 proteolysis was not observed in cells depleted of HCN2. Furthermore, the cleavage of AIF was also suppressed as a result of downregulation of HCN2 (Figure 3B). Accordingly, the mitochondrial liberation of AIF–GFP upon STS treatment was inhibited in cells with downregulated HCN2, and AIF remained in the mitochondria (Figure 3C). Nuclear localization of AIF in HCN2-expressing cells was confirmed using confocal microscopy (Supplementary Figure S9B). Furthermore, nuclear translocation of AIF was suppressed in cells where HCN2 was downregulated (Figure 3D). To confirm that the calpain–AIF signalling pathway was in fact responsible for cell death in this experimental model, four different methods were used. First, FACS analysis of Annexin V/PI-stained cells pre-exposed to either the pan-caspase inhibitor (zVAD-fmk.), the selective calpain inhibitor (PD150606), or siRNA against AIF was performed (Figure 3D and E). Second, condensed nuclei were counted under the same conditions (Supplementary Figure S6). Third, processing/activation of caspases-2, -3, -8 -9 and cleavage of PARP were monitored (Supplementary Figure S7). Finally, caspases-3/-7-like activity was measured (Supplementary Figure S7). In line with previous observations, all these results confirmed that the HCN2-mediated influx of Ca^{2+} triggered caspase-independent, AIF-mediated apoptosis.

Expression of HCN2 sensitizes HEK293 cells to STS/PKC412-evoked Ca^{2+} influx and apoptosis

HEK293 cells are deficient of HCN channels and, therefore, are commonly used as a model system to study heterologus expression (Yu *et al*, 2004). As expected, no HCN2 protein was detected using immunocytochemistry. Moreover, neither STS, nor PKC412, could stimulate Ca^{2+} influx into these cells (Figure 4A). Hence, we investigated if an STS/PKC412-evoked Ca^{2+} response and subsequent apoptotic effects could be introduced by expressing the HCN2 channel in HEK293 cells. Indeed, a prolonged PKC inhibitor-induced Ca^{2+} influx was observed in transfected cells harbouring the ectopic HCN2 channel (Figure 4B). As a control for the expression of HCN2, cells were fixed and immunostained for HCN2 (Figure 4A and B, right panels). Similar to previous observations, AIF was released from the mitochondria upon STS exposure, only in cells expressing HCN2 (Figure 4C). Next, when cells were exposed to the kinase inhibitors and analysed by FACS using Annexin V/PI staining, we observed that HEK293 cells survived STS or PKC412 treatment for at least 24 h (Figure 4D). However, morphological signs of cytotoxicity were detected, suggesting that they might die in a delayed manner. Conversely, the expression of HCN2 could sensitize these cells to STS/PKC412. These data demonstrate that HCN2 was indeed responsible for cellular Ca^{2+} influx after treatment with PKC inhibitors.

Dephosphorylation of Thr^{549} is essential for the prolonged Ca^{2+} entry through the HCN2 channel

To investigate whether the observed kinase inhibitor-induced Ca^{2+} influx was regulated by a phosphorylation-dependent mechanism, we exposed the cells to PKC activators. Pretreatment of cells with either one of the two different PKC activators (TPA or PMA), before administration of STS or PKC412, prevented the prolonged Ca^{2+} import through the

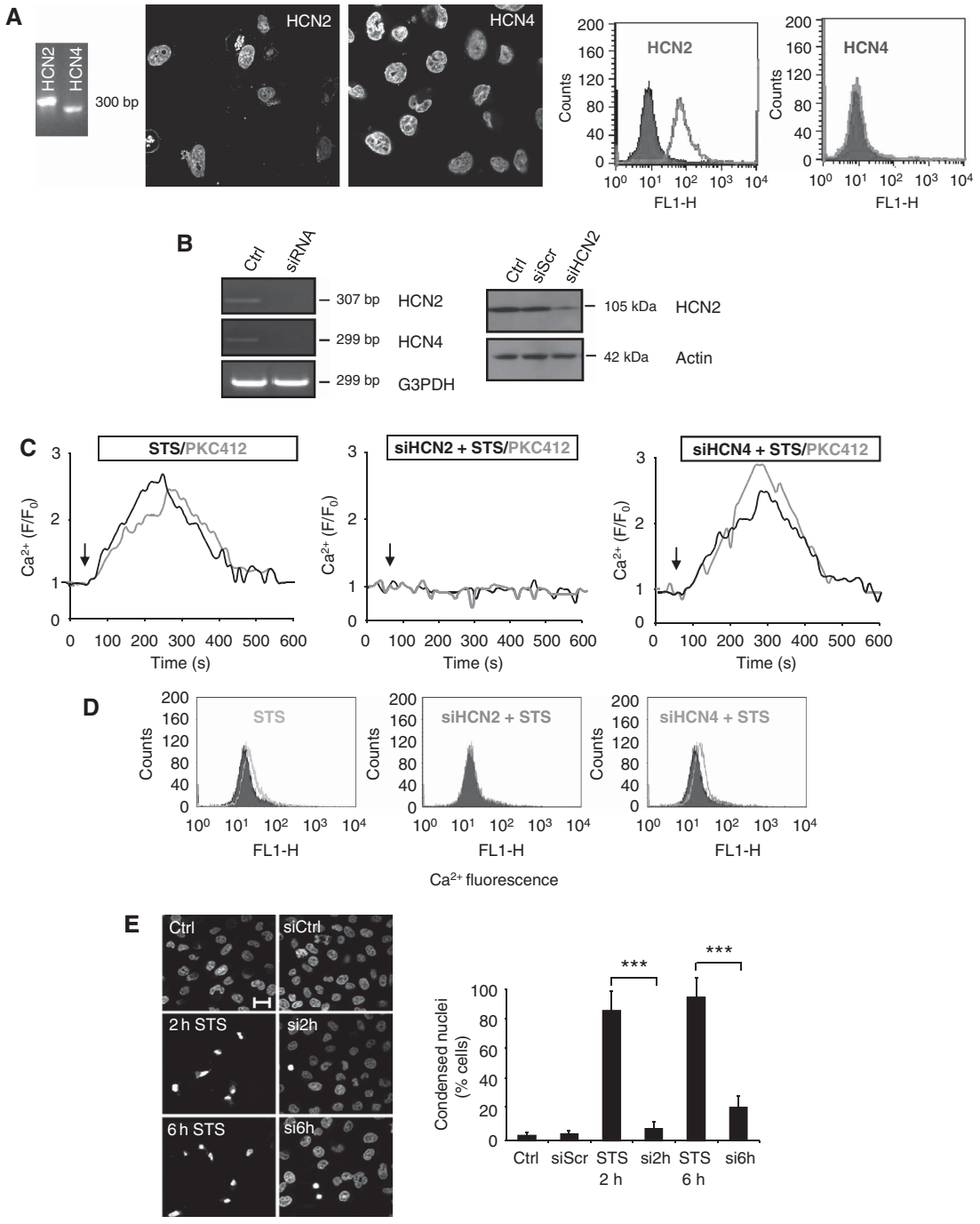

Figure 2 HCN2 channels mediate the PKC inhibitor-induced Ca^{2+} influx and subsequent cell death. (**A**) RT–PCR, immunostainings and FACS analysis of HCN2 and HCN4 expression. Cells were counterstained with DAPI. (**B**) mRNA level (left panel) and protein level (right panel) after 48 h of 100 nM siRNA silencing of HCN2 and HCN4 (**C**) Representative single-cell Ca^{2+} recordings of Fluo-4/AM-loaded cells exposed to 0.2–1 μM STS or 1–5 μM PKC412 in the presence or absence of HCN2 and HCN4. Ratio F/F_0 represents fluorescence of baseline. (**D**) FACS analysis of 10 000 Fluo-4-loaded cells with downregulated HCN2 or HCN4. (**E**) Cells were exposed to STS for different time periods (indicated in figure), fixed and the nuclei were stained with DAPI. Condensed nuclei were counted and presented in the graph as mean ± s.d. ($n = 4$). ***$P < 0.001$. Scale bar, 15 μm. A full-colour version of this figure is available at *The EMBO Journal* Online.

HCN2 channel (Figure 5A and B and Supplementary Figure S8). Next, the Ser/Thr phosphorylation level of the HCN2 in open/closed states was analysed. The results revealed that the channel is phosphorylated in control cells, and that exposure to STS for only 2 min led to significant dephosphorylation of the channel (Figure 5C). Furthermore, pretreatment of the cells with a PKC activator (PMA) suppressed this decrease. *In silico* analysis of the HCN2 channel revealed four putative PKC phosphorylation sites within its internal C-terminus domain. Alignment of human and mouse HCN2 sequences shows that these residues are conserved among species and suggests that the regulation of channel activity by phosphorylation might also be conserved (Figure 5D). Site-directed mutagenesis of each of the putative phosphorylation sites was performed, in which Ser/Thr was substituted with Ala. The various mutants were transfected into HEK293 cells, and as expected, record-

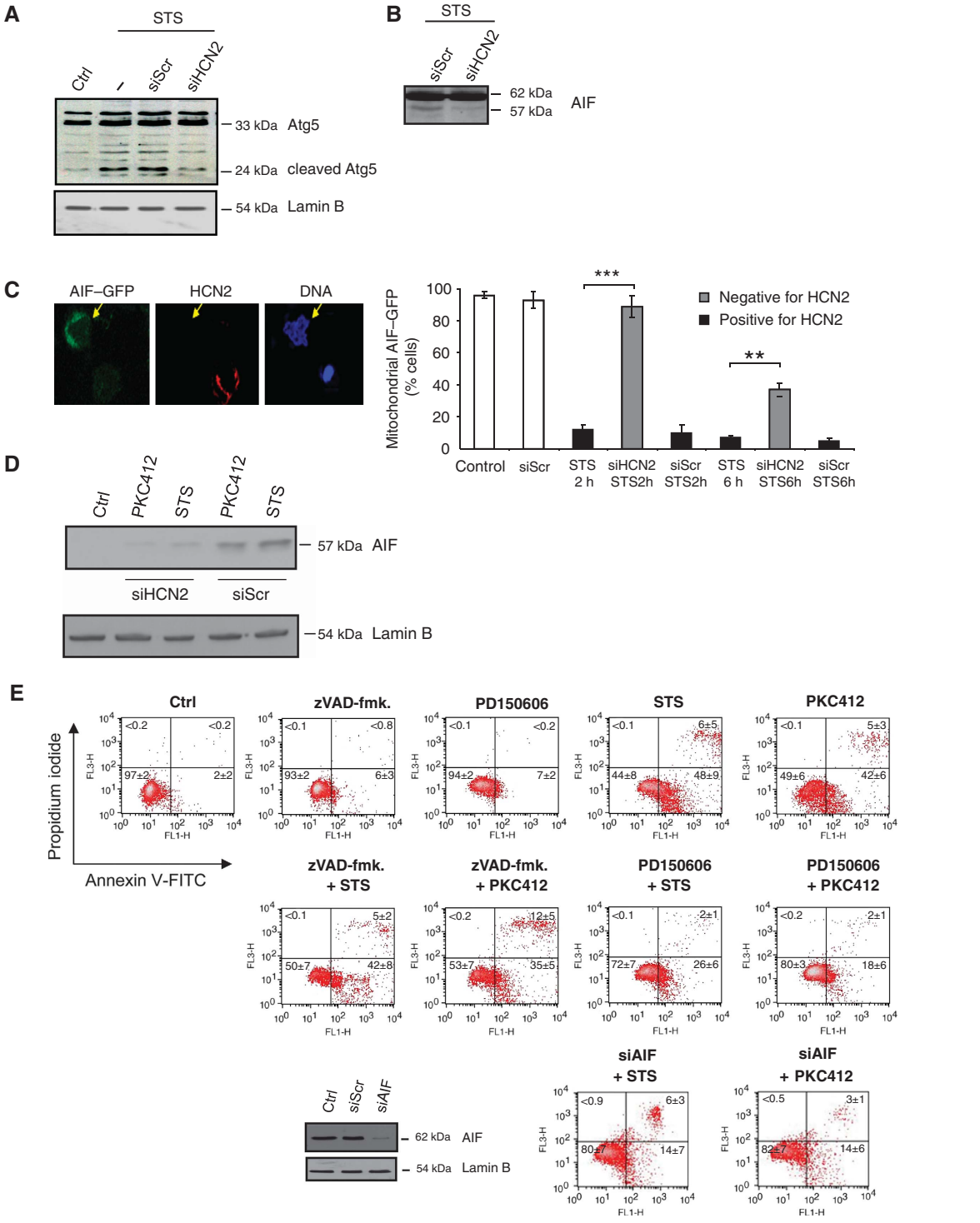

Figure 3 Ca^{2+} influx through HCN2 channels triggers caspase-independent AIF-mediated cell death. (**A**) The calpain-mediated cleavage of Atg5 in the presence or absence of HCN2 was analysed by western blot. The membranes were reprobed for Lamin B to confirm equal protein loading. (**B**) HCN2 was downregulated by siRNA, and cells were exposed to STS. AIF processing was assessed by western blot. (**C**) HCN2 was downregulated by an siRNA approach, and 24 h later cells were transfected with AIF–GFP. Cells were subsequently treated with STS for 2 and 6 h and fixed. Cells stained positively/negatively for HCN2 were analysed by immunostaining, and nuclei were counterstained with DAPI. A quantification of the amount of cells harbouring AIF–GFP in mitochondria after STS treatment is presented in the graph as mean ± s.d. ($n = 5$). $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t*-test) (**D**) Nuclei were isolated from control and HCN2-depleted cells exposed to STS/PKC412. The presence of AIF was analysed by western blotting. Membranes were reprobed for Lamin B to confirm equal loading of samples. (**E**) FACS analysis of Annexin V/PI-stained cells that were pretreated with either the pan-caspase inhibitor (10 μM zVAD-fmk.), a calpain inhibitor (150 μM PD150606) or siRNA against AIF before STS or PKC412 exposure. Downregulation of AIF was confirmed by western blot analysis. Membranes were reprobed for Lamin B to confirm equal loading of the samples.

ings of intracellular Ca^{2+} level showed that the four mutants responded to STS or PKC412 in a similar manner as wild-type cells (non-mutated plasmid) (Figure 5E).

The wild-type HCN2 channel is activated by hyperpolarization and dephosphorylation triggered by PKC inhibitors. Alternatively, the Ala mutants might be opened either by the same mechanism

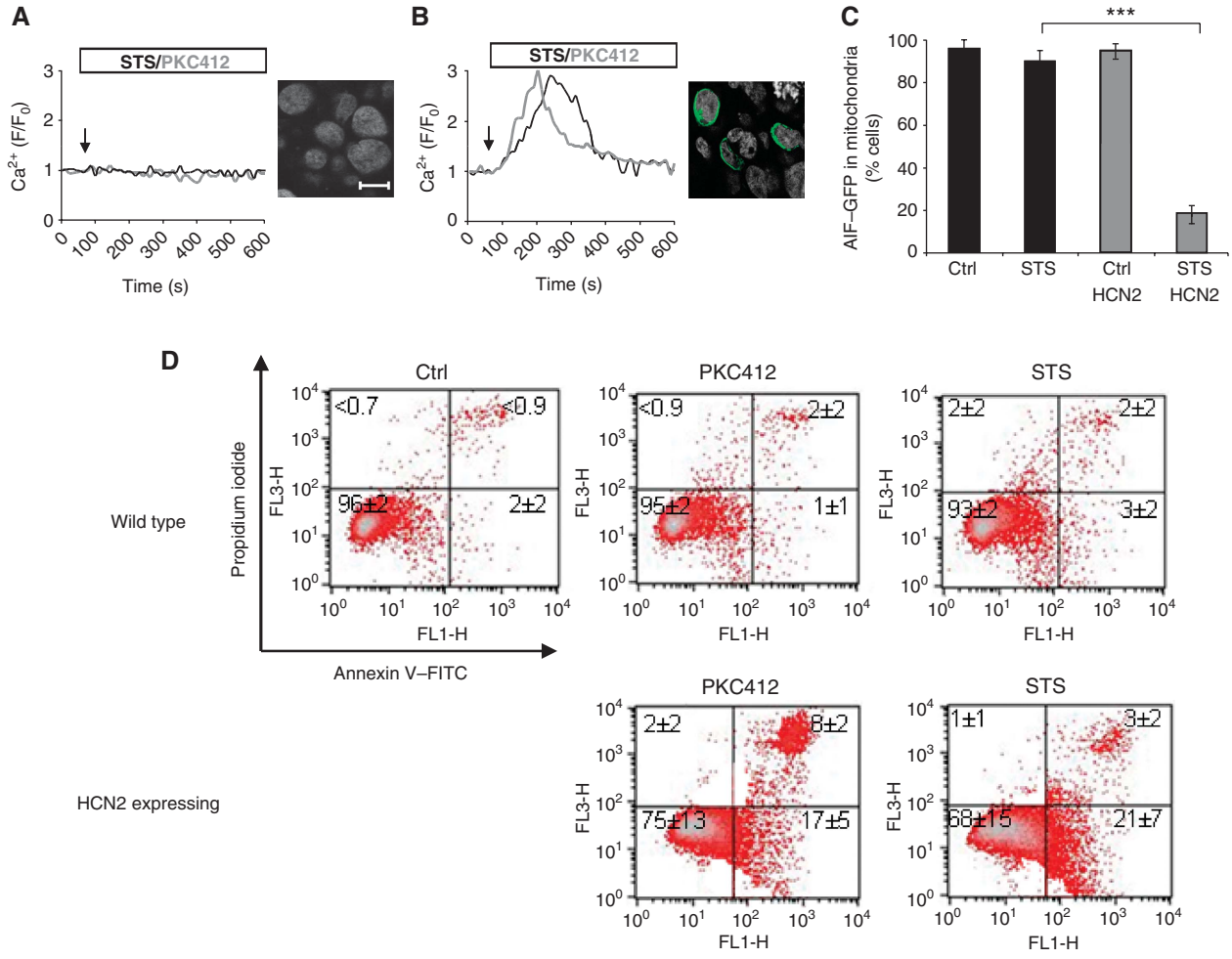

Figure 4 Expression of mHCN2 sensitizes HEK293 cells to STS/PKC412-induced Ca^{2+} influx and apoptotic cell death. (**A**) HEK293 cells were loaded with the Fluo-4/AM dye and exposed to STS or PKC412. Representative single-cell Ca^{2+} responses are shown in the graph. (**B**) The HCN2 channel was expressed in HEK293 cells, which were subsequently treated as in (**A**). (**C**) Control or HCN2-expressing HEK293 cells were transfected with AIF–GFP and exposed to STS. Cells were fixed and immunostained for HCN2. A quantification of cells with mitochondrial AIF–GFP is presented in the graph. Results are presented as mean ± s.d. ($n = 6$) (**D**) Control or HCN2-expressing HEK293 cells were exposed to STS/PKC412 and subsequently analysed by FACS for Annexin V/PI staining. Ratio F/F_0 represents fluorescence of baseline. ***$P < 0.001$ (Student's *t*-test). Scale bar, 15 μm.

or simply due to the hyperpolarization, as they mimic a dephosphorylated state of the channel. To test this possibility, HEK293 cells transfected with the different mutants were hyperpolarized by decreasing the $[K^+]$ level in the incubation medium (Figure 5F). These experiments revealed that the wild-type cells, as well as the Thr^{531}, Thr^{566} and Ser^{818} mutants, responded to the hyperpolarization with a transient Ca^{2+} increase, similar to what was observed using pretreatment with PKC activators, whereas a prolonged Ca^{2+} elevation was observed with the Thr^{549} mutant (Figure 5G). Thus, our results suggest that dephosphorylation of the HCN channel on residue Thr^{549} is pivotal for the prolonged Ca^{2+} influx mediated by the HCN2 channel upon treatment of NCSLC cells with PKC inhibitors.

PKC inhibition leads to the influx of Ca^{2+} through the HCN2 channel also in cortical neurons

Current *in vivo* data suggest that the AIF-mediated pathway might be particularly important for neuronal cell death (Zhu *et al*, 2007a, b; Hangen *et al*, 2010). Hence, we decided to investigate whether a mechanism similar to that observed in NSCLC cells might operate in primary cultures of rat cortical neurons. Similar to NSCLC cells, STS or PKC412 exposure resulted in a sustained increase in the intracellular Ca^{2+} level (Figure 6A). The Ca^{2+} influx was blocked by pre-incubation with Cs^+ (Figure 6B). Next, the HCN2 protein was downregulated using the siRNA approach (Figure 6C); this treatment prevented PKC inhibitor-induced Ca^{2+} entry (Figure 6D) and the subsequent processing of AIF (Figure 6E). Furthermore, by assessing the number of cortical neurons with condensed nuclei upon treatment with STS, we found that siRNA depletion of the HCN2 channel did suppress cell death also in this model system (Figure 6F). In summary, our results reveal that PKC inhibitor-induced Ca^{2+} import through the HCN2 channel is sufficient to trigger apoptosis also in cortical neurons and suggest that this might be a general mechanism of AIF activation in cell types expressing this channel.

Discussion

Intracellular Ca^{2+} overload can lead to apoptotic as well as necrotic cell death, and can be due to excessive Ca^{2+} entry from the extracellular milieu, impaired Ca^{2+} extrusion or inappropriate Ca^{2+} release from intracellular stores. Excess Ca^{2+} can cause cytotoxicity and cell death by the activation of a variety of

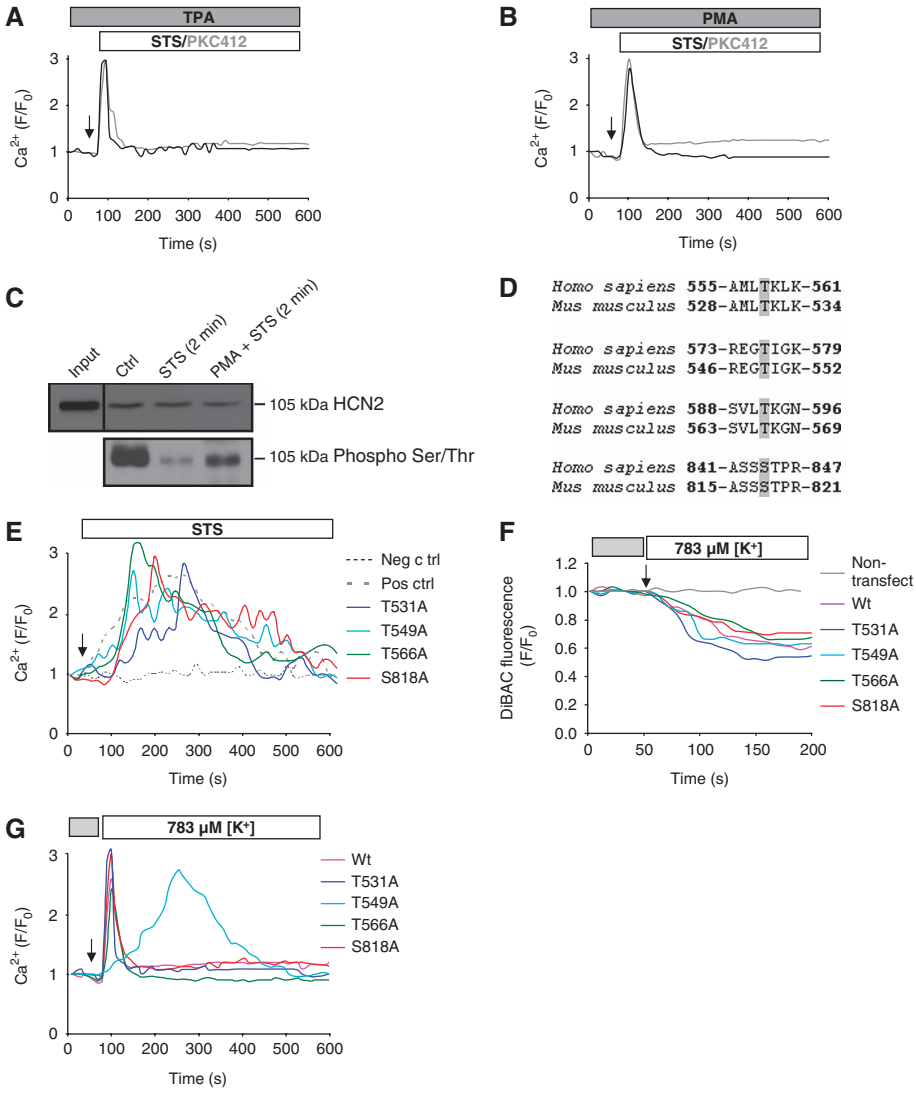

Figure 5 Dephosphorylation of Thr^{549} is essential for the prolonged Ca^{2+} influx through HCN2. U1810 cells were pretreated with the PKC activators (**A**) 1 µM TPA or (**B**) 300 nM PMA, before exposure to STS or PKC412. (**C**) The HCN2 channel was immunoprecipitated and analysed by western blot for phosphorylation on Ser/Thr residues. (**D**) Conserved putative PKC phosphorylation sites in the C terminal of human and mouse HCN2 are marked in grey. (**E**) The mutant and wild-type forms of the HCN2 channel were expressed in HEK293 cells, which were subsequently loaded with Fluo-4/AM, and the intracellular Ca^{2+} level was monitored upon exposure to STS. Untransfected HEK293 cells served as negative control and cells transfected with wild-type HCN2 channel was used as a positive control. MitoDsRed was used as a transfection marker. Hyperpolarization was induced by decreasing the K^+ level from 4.7 mM (grey box) to 783 µM (white box), and polarization was monitored using DiBAC (**F**) and the Ca^{2+} level using Fluo-4/AM (**G**) in HEK293 cells transfected with wild-type and various mutants of HCN2. Untransfected HEK293 cells served as negative control.

degradative enzymes, including Ca^{2+}-dependent proteases, or by impairment of mitochondrial function (Orrenius *et al*, 2003).

NSCLC cells are resistant to conventional anticancer treatments, but can be killed by the PKC inhibitors, STS and PKC412. The responsible cell death pathway involves the influx of extracellular Ca^{2+}, leading to calpain-mediated mitochondrial processing and nuclear translocation of AIF, which causes chromatin condensation, DNA fragmentation and apoptotic cell death (Joseph *et al*, 2002; Norberg *et al*, 2008, 2010a, b). However, the precise regulation of this mechanism and the identity of the plasma membrane channels responsible for the Ca^{2+} entry remain elusive.

Recently, it was reported that two of the HCN channels previously identified in the plasma membrane of multiple cell types are also permeable to Ca^{2+}, in addition to K^+/Na^+ (Yu *et al*, 2004; Michels *et al*, 2008). This finding suggested a possible role for these channels in mediating cellular Ca^{2+} influx. Unlike most other plasma membrane Ca^{2+} channels, HCN channels are activated by hyperpolarization. By monitoring polarization of the plasma membrane, we found that treatment of NSCLC cells with STS or PKC412 resulted in hyperpolarization of the plasma membrane and Ca^{2+} influx into the cells (see Figure 1A, 2B and C). It was reported previously that the Ca^{2+} current flow through the HCN2 channel triggered by hyperpolarization pulses is rather slow in comparison with that seen in other Ca^{2+} channels (Yu *et al*, 2004). In line with these reports, we observed a time-dependent, sustained Ca^{2+} influx through the HCN2 channel that was followed by a normalization of the Ca^{2+} level (see Figure 1A). The imported Ca^{2+} caused an elevation of the intracellular Ca^{2+} level that persisted for several minutes, which was sufficient to trigger apoptosis through activation

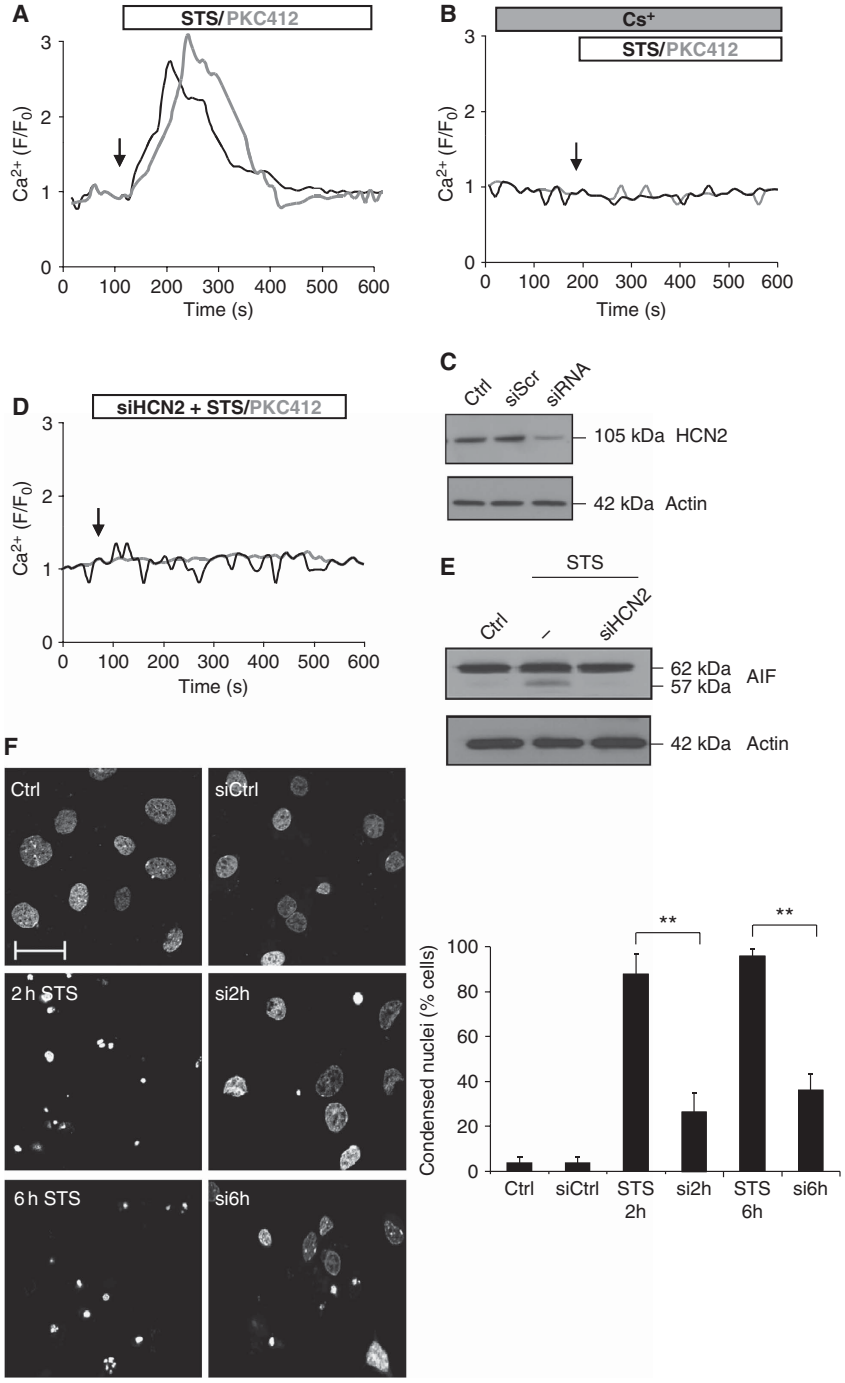

Figure 6 PKC inhibition leads to the entry of Ca^{2+} through the HCN2 channel in primary culture of cortical neurons. The cells were loaded with Fluo-4/AM and exposed to (**A**) STS or PKC412 and (**B**) CsCl before STS or PKC412. (**C**) Western blot analysis of the HCN2 level after siRNA-induced downregulation. HCN2 was downregulated by siRNA and cells were either (**D**) loaded with Fluo-4/AM and exposed to STS or PKC412 or (**E**) exposed to STS followed by western blot analysis of AIF cleavage. (**F**) Cells were exposed to STS for different time periods (indicated in figure), fixed and the nuclei were stained with DAPI. Condensed nuclei were counted and presented in the graph as mean ± s.d. ($n = 4$). $^{**}P < 0.01$. Scale bar, 15 μm.

of the calpain–AIF axis (see Figure 2E, G and H and Supplementary Figure S6). All these apoptotic events occurred in a time window where caspase activation was not yet detected (Supplementary Figure S7).

Opening of HCN channels is regulated by cAMP binding and phosphorylation-dependent mechanisms. For instance, tyrosine kinases of the Src kinase family have been shown to phosphorylate HCN1, HCN2 and HCN4, resulting in a more rapid activation of the channel (Zong *et al*, 2005; Biel *et al*, 2009). Moreover, inhibition of Ser/Thr p38 MAP kinase has been reported to cause hyperpolarization and activation of the HCN channel (Poolos *et al*, 2006). Our results suggest that there is a constitutive phosphorylation by PKC of Thr^{549} within the conserved C-terminal regulatory domain of the channel, and that the PKC inhibitors, PKC412 or STS, trigger both hyperpolarization and dephosphorylation of Thr^{549}. Hence, hyperpolarization alone would only trigger a transient Ca^{2+} influx through the HCN channel. Similarly, when PKC

activators are present to prevent dephosphorylation events, STS/PKC412 could only stimulate a transient Ca^{2+} response (see Figure 5A and B). Such a Ca^{2+} transient has been reported earlier to be insufficient to activate an apoptotic response (Norberg *et al*, 2008). However, when Thr^{549} was mutated and could no longer be phosphorylated, hyperpolarization *per se* resulted in a prolonged Ca^{2+} elevation in the absence of any drug exposure of cells. Hence, it appears that the dephosphorylation of Thr^{549} is pivotal for the involvement of HCN2 in cell death signalling.

There are multiple isoforms of PKC, including PKC-α and PKC-epsilon, which have been reported to promote proliferation and survival of NSCLC cells. These PKC isoforms were also found to be overexpressed in NSCLC. Therefore, protein kinases C are attractive drug targets, and several PKC inhibitors have been designed that are currently being investigated in clinical trials (Reyland, 2009). Our results support this approach, as exposure of NSCLC cells to either one of the PKC inhibitors, STS or PKC412, led to intracellular Ca^{2+} elevation and subsequent AIF-mediated cell death. In particular, PKC412 is clinically relevant as it is currently used in combination with Cisplatin and Gemcitabine for the treatment of patients with NSCLC, and it has been reported to interact strongly with PKC-α, -β and -γ (Reyland, 2009). Moreover, PKC412 was shown to have greater antitumour activity in nude mice bearing human H460 NSCLC tumour xenografts as compared with other conventional cytotoxic agents, including doxorubicin, cyclophosphamide, cisplatin and gemcitabine (Monnerat *et al*, 2004). Our findings might therefore have particular implications for treatment of NSCLC.

Materials and methods

Cell culture and transient transfection

U1810 and H661 NSCLC cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and 2% (w/v) glutamine. HEK293 cells were grown in DMEM medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. Cells were grown in a humidified 5% CO_2 atmosphere at 37°C at a cell density allowing exponential growth. For the plasmid transfections, cells were seeded on coverslips and on the following day transfected with either pEGFP–AIF or pcDNA3.1–mHCN2 using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instruction. pMitoDsRed was used as a transfection marker.

Culture of cerebral cortical neurons

Primary cultures of cerebral cortical neurons were prepared from Sprague–Dawley rat pups, 1–3 days old. The cerebral cortices were dissected in Hank's balanced salt solution (HBSS). HBSS was removed and the tissues were treated with 0.25% trypsin at 37°C for 15 min. This was followed by a 5 min treatment with 0.1% DNase I at 37°C. Cells were then diluted to 7×10^5 cells/ml in DMEM medium containing 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mmol/l L-glutamine, and were seeded on plates precoated with 50 μg/ml poly-L-lysine. After 3 h, DMEM was replaced with neurobasal medium (GIBCO) containing B27 Supplement (GIBCO), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mmol/l L-glutamine. Half of the cell culture medium was replaced after three DIV with neurobasal medium containing the supplements specified above and 10^{-7} mol/l aracytine.

Immunocytochemistry

Cells were seeded on coverslips, fixed for 20 min in 4% formaldehyde at 4°C and then washed with PBS. Incubations with primary antibodies diluted (1:400) in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA) were performed at 4°C overnight. The slides were washed the following day with PBS before incubation with secondary antibodies (1:200) at room temperature for 1 h. Nuclei were counterstained with DAPI (10 μg/ml) by 5 min incubation at room temperature. The following primary antibodies were used: rabbit anti-HCN2 (Alomone), goat anti-HCN2 and goat anti-HCN4 (Santa Cruz Biotechnology). Secondary FITC-conjugated antibodies (Molecular probes) were directed to goat (Alexa 488). Stained slides were mounted using Vectashield H-1000 (Vector Laboratories Inc.) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Zeiss).

Immunoblotting

Equal amount of the protein samples were mixed with loading buffer, boiled for 5 min, and subjected to 15% SDS–PAGE at 40 mA followed by transfer to nitrocellulose membranes for 90 min at 120 V. Membranes were blocked for 30 min with 5% non-fat milk in TBS at room temperature and subsequently probed with rabbit anti-cleaved Atg5 (Abgent), goat anti-AIF (Santa Cruz Biotechnology), rabbit anti-actin (Sigma) or rabbit anti-HCN2 (Alomone). ECL™ (Amersham Biosciences) was used for revealing the blots. The primary antibodies were diluted in TBS containing 1% BSA, 0.05% Tween-20 and 0.1% NaN_3. Horseradish peroxidase-conjugated secondary antibodies (Pierce) were diluted in 2.5% blocking buffer.

Live cell imaging

To perform Ca^{2+} or polarization measurements the cells were incubated (30 min at 37°C in 5% CO_2) in KREBS medium containing 5 μM Fluo-4/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes) or 150 nM DiBac. The Hepes medium contained 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 20 mM Hepes (pH 7.4) and 5 mM dextrose. Hyperpolarization that was induced by decreasing the extracellular K^+-level was performed as follows. Cells were incubated in 500 μl KREBS medium and subsequently the K^+ concentration was decreased to 17% (783 μM) by the addition of K^+-free KREBS. Images were acquired at 0.2 Hz. During the time-lapse experiments all drugs were bath-applied and examined under a Zeiss LSM 510 META scanning laser confocal microscope (Zeiss). The following drugs were used: 0.2–1 μM STS and 1–5 mM PKC412, 1 μM TPA and 300 nM PMA.

Flow cytometry

Cells were loaded with DiBac or Fluo-4/AM as described above and treated with 1 μM STS or 3 mM PKC412 immediately before FL-1 FACS analysis of 10 000 cells. Cell death was assessed using the Annexin V-FLUOS staining kit (Roche), according to the manufacturer's instruction.

For analysis of HCN2 and HCN4 staining, cells were washed in PBS and fixed with 0.25% paraformaldehyde for 10 min at room temperature. Next, cells were stained with HCN2 and HCN4 antibodies as described in Materials and methods section, and Immunocytochemistry.

RNA isolation and RT–PCR

Total RNA was isolated from U1810 or H661 cells using the RNeasy kit (Qiagen). The quality of the RNA was controlled by gel electrophoresis of 18S and 28S ribosomal RNA. For the first-strand cDNA synthesis, the RevertAid™ M-MuL V RT enzyme (Fermentas) was used in combination with gene-specific primers according to the manufacturer's instruction. The PCR was performed using the Platinum®Pfx DNA polymerase system (Invitrogen) and the following specific primers (Invitrogen): HCN2 (forward primer, CGGCCCAGTGGCTGAGAGGA; reverse primer, CACTGCTGCCTCG GCTCGTC), HCN4 (forward primer, GGCCACTTCCACAAGGCGCT; reverse primer, CACAAGGGACGGCGGCTCAG), G3PDH (forward primer, CCTGGCCAAGGTCATCCATG; reverse primer CTGACACGTT GGCAGTGGG). The conditions for HCN2 and G3PDH PCR reactions were as follows: 94°C for 1 min (hot start) followed by 95°C for 1 min, 55°C for 30 s and 72°C for 1 min. For the PCR of HCN4 57°C was used as annealing temperature. Samples were removed every fifth cycle to ensure exponential growth of the PCR product, which was then analysed on a 1% agarose gel.

Measurement of caspases-3/-7-like activity

The measurement of caspase substrate (Peptide Institute, Osaka, Japan) cleavage was performed as follows: 2×10^6 cells were washed once with PBS, resuspended in 25 μl of PBS and placed on a microtiter plate. Cells were subsequently mixed with DEVD-AMC

(50 μM), dissolved in standard reaction buffer (100 mM Hepes, pH 7.25, 10% sucrose, 10 mM DTT, 0.1% CHAPS). Cleavage of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan II plate reader (Thermo Electron Co., Waltham, MA, USA) using 355 nm excitation and 460 nm emission wavelengths.

siRNA approach

Silencing of HCN2, HCN4 and AIF expression was achieved by transfection of anti-HCN2 (ONTARGETplus SMARTpool L-006201-00-0005, Dharmacon), anti-HCN4 (ONTARGETplus SMARTpool L-006203-00-0005, Dharmacon) and anti-AIF (PDC8) (ONTARGETplus SMARTpool L-011912-00-0005, Dharmacon) using INTERFERIN siRNA transfection reagent (Polyplus transfection). The level of the mRNAs was monitored by RT–PCR and the level of protein by western blot.

Site-directed mutagenesis

The pcDNA3.1–mHCN2 plasmid was kindly provided by Prof Hang-Gang Yu, West Virginia University School of Medicine, Morgantown. Quick-change II XL Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's instructions to generate the mutations of putative PKC phosphorylation sites within the regulatory C-terminal domain of HCN2: T531A, T549A, T566A, S818A. The following primers were used: T531A (forward primer, TCACAGCCATGCTGGCAAAGCTCAAATTTG; reverse primer, CAAATTTGAGCTTTGCCAGCATGGCTGTGA), T549A (forward primer, CCGAGAGGGGGCCATCGGGAAGA; reverse primer, TCTTCCCGATG GCCCCCTCTCGG), T566A (forward primer, TGAGCGTGCTCGCCAA GGGCAACAA; reverse primer, TTGTTGCCCTTGGCGAGCACGCTCA), S818A (forward primer, GGCCAGCAGCGCCACGCCGC; reverse primer, GCGGCGTGGCGCTGCTGGCC). Desired substitutions were confirmed by sequencing.

Statistical analysis

Data from at least three independent experiments are presented as mean ± s.d. Student's unpaired *t*-test was used for the comparisons between different groups. A value of $P<0.05$ was considered as statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: EN, PU, SO and BZ designed research; EN, MK, OK performed research; SS performed electrophysiological recordings; EN, GS, PU, SO and BZ analysed data; and EN, PU, SO and BZ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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Bacillus Calmette-Guerin (BCG) Triggers PLC-Dependent Ca^{2+} Signaling in Bladder Cancer Cells

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Abstract

Bacillus Calmette-Guérin (BCG) is utilized as cancer therapy, mainly for urinary bladder cancer, with very good results for non-muscle-invasive bladder cancer. Despite the efficiency in killing the cancer little is known about the sub-cellular signaling pathways activated by BCG. Here we demonstrate that BCG inhibits urinary bladder cancer cell proliferation through a PLCβ3-dependent calcium (Ca^{2+}) signal. Experiments using Ca^{2+} free medium also showed Ca^{2+} responses, thus indicating that Ca^{2+} released from internal stores were responsible for this signaling event. Depleting the endoplasmic reticulum (ER) Ca^{2+} store with cyclopiazonic acid significantly blocked the BCG-induced Ca^{2+} response. Small interference RNA knock-down of PLCβ3 or pharmacological block with U73122 or ET-18-OCH_3 stopped Ca^{2+} signaling and reversed the inhibitory effect of BCG on cancer cell proliferation. Inhibition of pertussis toxin-sensitive G-protein receptors blocked the observed effect. Labeling BCG with a fluorescent marker indicated that BCG targeted to the ER structure of the cell. These results present new actions of BCG in triggering PLC-dependent Ca^{2+} signaling that prohibit cancer growth of urinary bladder cancer cells.

Introduction

Urinary bladder cancer is one of the ten most common cancer-types in the world (Jemal et al., 2009). The most frequent bladder cancer type is non-muscle invasive cancer, that represents 70-80 % of the cases (Simons et al., 2008). The initial treatment of non-muscle invasive cancer is transurethral resection (TUR). However, tumor recurrence occurs in up to three quarters of the patients after TUR and in some cases the tumor further progress to invasive muscle cancer (occurs in ~20 %) (Amling, 2001). In order to prevent this recurrence and progression, intravesical delivery of Bacillus Calmette-Guerin (BCG) is used. BCG was originally designed as an antituberculosis live attenuated vaccine prepared from *Mycobacterium bovis*, but has been used as an anticancer agent over three decades given the clinical observation of being more effective than classic chemotherapy and radiotherapy treatments (Kassouf and Kamat, 2004; Liu et al., 2009; Morales et al., 1976).

The cellular mechanisms in urinary bladder cancer cells activated by BCG are not clearly understood. It is believed that BCG give rise to local immune and inflammatory responses, which leads to recruitment of macrophages, neutrophiles, T cells, NK cells and dendritic cells, and to increased levels of cytokines and chemokines that in turn may account for the cytostatic effects of BCG (Horinaga et al., 2005; Kamat et al., 2009; Luo and Knudson, 2010). Reports also state that BCG causes NF-κB transcription activation in urinary bladder cancer cells (Chen et al., 2002).

The calcium ion (Ca^{2+}) is a ubiquitous intracellular second messenger that regulates a diverse range of cellular processes in physiological and pathological conditions (Berridge et al., 1998; Clapham, 2007). The temporal and spatial features of intracellular Ca^{2+} signals are controlled by channels and pumps that transport Ca^{2+} through the plasma membrane or between the cytoplasm and the endoplasmic reticulum (ER) (Berridge et al., 2003; Norberg et al., 2010). Release of Ca^{2+} from intracellular ER Ca^{2+} stores mainly occurs through the inositol 1,4,5-trisphosphate ($InsP_3$) receptors ($InsP_3Rs$) which are activated when PIP_2 is cleaved by Phospholipase C (PLC) into $InsP_3$ and diacylglycerol. The subsequent elevation of cytosolic Ca^{2+} concentration activates downstream effectors, including protein kinases and transcription factors (Desfrere et al., 2009; Norberg et al., 2010), which in turn regulates multiple cellular processes.

In this study we show that BCG elevates the cytosolic concentration of Ca^{2+} in human urinary bladder cancer cells. This signaling cascade involves PLCβ3 as pharmacological inhibition or small interference RNA-mediated gene silencing as well as ER store depletion abrogates the response. Our results also demonstrate that BCG blocks cancer cell proliferation in a Ca^{2+}- and PLC-dependent manner.

Results

BCG induces an intracellular Ca^{2+} response in human urinary bladder cells

Human bladder cancer cells (T24) were loaded with a Ca^{2+}-sensitive fluorescent dye Fluo-3/AM (Fig. 1A) to investigate how the Ca^{2+} homeostasis was affected by BCG. Untreated cells showed a stable baseline with little spontaneous Ca^{2+} activity (Fig. 1B). The viability of the cells under our experimental conditions was tested by exposing the cells to an agonist towards the end of the experiment. Indeed ATP, a purinergic receptor agonist, triggered a prominent Ca^{2+} transient after recording a baseline for 30 minutes (Fig. 1C). Treating the cells with BCG rapidly induced a Ca^{2+} response that could be divided into an oscillating, transient, or sustained response (Fig. 1D and 1H) (responses see supplementary figure 1). The contribution of extracellular Ca^{2+} was next tested by removing Ca^{2+} from the extracellular medium. The BCG stimulated Ca^{2+} response still remained in the absence of extracellular Ca^{2+} (Fig. 1E and 1H), indicating the intracellular Ca^{2+} was the source. Also the renal cell carcinoma line KU-19-20 responded with elevated Ca^{2+} following BCG (Fig. 1F), thus showing that the observed effect was not unique to T24 cells. It is known that peptidoglycan (PGN), a polymer found in the bacterial cell wall (Rogers HJ, 1980) consisting of N-acetylglucosamine and N-acetylmuramic acid, can stimulate Ca^{2+} signaling in macrophages and dendritic cells through PLCγ2 (Aki et al., 2008). Applying PGN to T24 cells evoked Ca^{2+} oscillations similar to those triggered by BCG (Fig. 1G). These data show that BCG can stimulate cytosolic Ca^{2+} signaling that partially originates from release of Ca^{2+} from internal stores.

Thereafter BCG was labeled with the fluorescent non-nucleic BacLight Red stain to examine the binding target in the cell. Using a confocal microscope BacLigth stained-BCG were shown to interact with a sub-cellular target located outside the nucleus (Fig. 2A), resembling the ER structure. Therefore cells were double-stained with the ER marker ER-Tracker (Fig. 2B) and imaged simultaneously with BacLight-stained BCG. These results revealed a co-localization between BacLight-stained BCG and ER (Fig. 2D).

PLC is affecting BCG induced Ca^{2+} singling

Pharmacological experiments were conducted to examine the downstream cellular signaling pathway of BCG. Pre-incubation with the Ca^{2+} chelator BAPTA suppressed the BCG-induced Ca^{2+} response (Fig. 3A). Cyclopiazonic acid (CPA) that blocks the sarcoplasmic/endoplasmic reticulum calcium (SERCA) pump was used to investigate the involvement of intracellular ER Ca^{2+} stores in BCG-induced Ca^{2+} signaling. CPA caused a transient Ca^{2+} increase as internal ER stores were emptied (Fig.

3B). Depleting ER stores significantly suppressed the Ca^{2+} response triggered by BCG (Fig. 3B and I). 2-Aminoethoxydiphenyl borate (2-APB) was next used to inhibit $InsP_3Rs$. Following pre-treatment with 2-APB reduced the number ofcells responding to BCG (Fig. 3D).

PLC is involved in the production of $InsP_3$, which stimulates Ca^{2+} release from the $InsP_3R$. Therefore PLC was blocked with the two different PLC inhibitors U73122 and ET-18-OCH_3. Both these inhibitors significantly reduced the BCG-induced Ca^{2+} response (Fig. 3D, E, and I). In contrast, U73122´s inactive analogue U73343 failed to block the Ca^{2+} response (Fig. 3F and I). Also the involvement of phosphatidylinositol 3-kinases (PI3Ks), an enzyme family involved in proliferation and cell growth, was investigated. Inhibiting PI3Ks with Wortmannin did not influence the BCG-triggered Ca^{2+} response (Fig. 3G and I). Preincubation with the G-protein coupled receptor (GPCR) inactivator Pertussis toxin (PTX) for 6 h prior BCG application suppressed the increase in Ca^{2+} (Fig. 3H and I).

Small interfering RNA (siRNA) against PLC was used to further elucidate the signaling pathway activated by BCG. PLC can provoke intracellular Ca^{2+} increase through $InsP_3$ production via two major pathways. One pathway involves PLCβ that is activated through GPCR, while the other pathway involves PLCγ that is activated by receptor tyrosine kinase (RTK). Four different isoforms of PLCβ exist, of which PLCβ2, PLCβ3 and PLCβ4 were expressed in T24 cells (Fig. 4A), as shown with RT-PCR. Since PLCβ3 is the key player in Ca^{2+} signaling (Hwang et al., 2005; Le Mellay and Lieberherr, 2000; McCullar et al., 2007) that isoform was further investigated using siRNA. Western blot analyses revealed a reduced protein level after 24 and 36 h with PLCβ3-siRNA (Fig. 4B). The knockdown of PLCβ3 significantly suppressed BCG-induced Ca^{2+} signaling (Fig. 4C and D). In contrast, knocking down PLCγ with siRNA (Fig. 4E) failed to abolish the Ca^{2+} response (Fig. 4F).

Taken together these data suggest that PLCβ3 plays a major role in BCG stimulated Ca^{2+} signaling in urinary bladder cancer cells.

The supernatant of BCG also stimulates Ca^{2+} singling

The BCG mixture introduced by catheter into the patient's bladder is diverse containing whole bacteria plus bacterial fragments from external cell wall, intracellular content, as well as smaller particles secreted from the bacteria itself. The supernatant of BCG (sBCG) was also tested on urinary bladder cancer cells. sBCG provoked a Ca^{2+} response in T24 cells (Fig. 5A and I). The sBCG response very much resembled the BCG response besides that fewer cells were oscillating when treated with sBCG (Fig. 5I). Also sBCG evoked a response independent of external Ca^{2+} (Fig. 5B and H). Preloading cells with BAPTA abolished the response (Fig. 5C and I). Inhibiting PLC with U73122 (Fig. 5D and I) or

ET-18-OCH_3 (Fig. 5E and I) reduced the ability of sBCG to elevate cytosolic Ca^{2+}. The inactive U73433 had no effect on the response (Fig. 5F). Preincubation with PTX also suppressed the sBCG-induced Ca^{2+} increase (Fig. 5G and I). These data demonstrated that sBCG has a similar effect on Ca^{2+} signaling as BCG.

BCG PLC-dependently affects cell proliferation

The dependency of BCG-induced Ca^{2+} signaling on proliferation of urinary bladder cancer cells was next studied using immunocytochemistry of Ki67 and EdU (Fig. 6A). Ki67 is present in nucleus of cells in all active cell cycle states (G1, S, G2 and M) but is absence in resting cells (G0), while EdU detects cells only in the s-phase of the cell cycle. Since it is known that the immune-response is involve in the positive effect from BCG therapy, DSMO-differentiated human promyelotic leukemia PLB-985 cells to mature neutrophil-like cells were added to the T24 cells (Ashkenazi and Marks, 2009). Treatment with sBCG, PLB-985 or PLB-985 in addition to sBCG had no effect on Ki67 after either 48 or 72 h (Fig. 6B). sBCG alone or in combination with PLB-985 cells did not either effect the amount of EdU positive cells (Fig. 6C). Neither BCG nor PLB-985 in addition to BCG had any effect on Ki67 expression (Fig. 6D). On the other hand when looking at EdU expression, BCG independent of PLB-985 cells showed a decrease in the percent of EdU positive cells following 72 h treatment (Fig. 6E). These results indicate that the cells still remained in the cell cycle after the sBCG treatment, but fewer cells reached the s-phase.

The involvement of PLC on BCG-altered cell proliferation was next examined. The PLC inhibitor U73122 and its inactive analogue U73343 were applied to the cells and after 72 h of treatment with BCG the expression of Ki67 and EdU were detected. Neither U73122 nor U73343 alone or in combination with BCG influenced the Ki67 expression (Fig. 7A and B). However, when U73122 was added together with BCG the effect on EdU was abolished while U73343 had no effect (Fig. 7A and C). Taken together the treatment with the PLC inhibitor U73122 reversed the BCG-induced effect on EdU, as previously observed. These results suggest an additional role for PLC in modulating cell proliferation.

Discussion

Here we show that treating urinary bladder cancer cells with BCG raises the cytosolic Ca^{2+} concentration and stops cell proliferation. This signaling event was dependent on PLCβ3 since pharmacological inhibition or small interference RNA-mediated gene silencing abolished the response. Store depletion by SERCA inhibition also blocked the BCG-triggered signal cascade. When cells were pre-treated with the GPCR-inhibitor pertussis toxin the response was blocked. Recoding EdU incorporation showed that BCG-controlled cell proliferation was mediated via a Ca^{2+}- and PLC-dependent mechanism.

Intravesical therapy with BCG vaccine is the most effective treatment for non-muscle-invasive urinary bladder cancer applied today and it has been used as a routine treatment for over the last 3 decades (Morales et al., 1976). BCG is given in addition to transurethral resection (TUR) and has been shown to reduce the risk for both progression and recurrence of the cancer. Despite the frequent usage of BCG in the clinic, little is known about its exact actions on cells. Most cellular studies are reporting that BCG starts a local immune response reaction in the bladder. The immune response involves recruitment and migration of a large variety of immune cells e.g.; macrophages, neutrophiles, B- and T-cells, natural killer cells, dendritic cells to the bladder and increased levels of secreted chemokines, interleukins and cytokines (Horinaga et al., 2005; Kamat et al., 2009; Liu et al., 2009). However, few reports on non-immune responses exist. In this study it is shown that BCG also triggers a non-immune response via Ca^{2+} signaling. A complete picture of BCG's actions is needed to optimize the treatment and reduce unwanted side-effects.

A major challenge with the BCG therapy is its unpleasant contraindications and side effects. Common contraindications are BCG sepsis, immune-suppression, heamaturia, active urinary tract infection and mild cystitis (Kresowik and Griffith, 2009). One other disadvantage with the therapy is the total failure in some of the patients (O'Donnell and Boehle, 2006). When the bladder cancer patients are given the BCG therapy they get intravesical installations with live attenuated *Mycobacterium bovis* BCG vaccine. These installations have a diverse content including whole bacteria plus fragments of bacteria in addition to all the components secreted from the bacteria itself. Most probably this diverse mixture has different effects on the cell. The data presented here show that Ca^{2+} signaling is activated by both the complete BCG as well as the supernatant of BCG. The exact effect of the BCG-induced Ca^{2+} signal in the intact bladder remains to be investigated. We hypothesize that there is a cross-talk between the non-immune Ca^{2+} signal and the immune response in the *in vivo* setting. Intracellular Ca^{2+} signaling represent an universal mechanism in cells that is involved in regulation of

a variety of processes like proliferation, differentiation, gene transcription and apoptosis (Berridge et al., 2003). Therefore we believe that BCG-stimulated Ca^{2+} signaling plays a crucial role in stopping the progress of cancer growth in urinary bladders. In the future combination treatments of BCG together with pharmacological regulators of the cellular Ca^{2+} homeostasis might increase the efficiency of the treatment.

Our data show that in addition to BCG also sBCG (the supernatant of centrifuged BCG) stimulates elevation of cytosolic Ca^{2+}. This Ca^{2+} response is observed in a similar percentage of cells treated with sBCG as with BCG. Furthermore, both BCG and sBCG are blocked by the same inhibitors. However, a clear distinction between the response pattern of BCG and sBCG is observed. BCG evoked more oscillating cells while sBCG stimulated less oscillating cells but more transient cells. This result could be explained by our experiment showing that peptidoglycan (PGN), a polymer in the bacterial cell wall, rather induces Ca^{2+} oscillations. Further investigations are required to resolve all the actions of the BCG mixture.

In this study it was demonstrated that BCG elevates the cytosolic concentration of Ca^{2+} in urinary bladder cancer cells. The signaling cascade was shown to be dependent on PLCβ3 and release of Ca^{2+} from internal ER Ca^{2+} stores. Our data also showed that BCG did bind a target inside the cell and that the supernatant of BCG as well could trigger a similar response. Inhibiting pertussis toxin-sensitive GPCRs blocked the BCG-induced Ca^{2+} response. Recoding EdU incorporation showed that BCG-controlled cell proliferation was mediated via a Ca^{2+}- and PLC-dependent signaling mechanism.

Material and Methods

Cell culture

Human T24 urinary bladder cells and human promyelotic leukemia PLB-985 cells were cultured in RPMI 1640 media containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 µg/ml streptomycin and 2 mmol/l L-glutamine and grown at 37°C with 5% CO_2.

BacLight staining

A 100 µM BacLight Red (Molecular Probes, Invitrogen) solution was diluted 1:1000 in the bacterial sample and incubated for 15 min at room temperature before addition to the cells and overnight incubation in 37°C with 5% CO_2. Cells were double stained with ER-tracker (Molecular Probes, Invitrogen) before being monitored using an Olympus FluoView 1000, confocal laser scanning microscope.

Calcium imaging

Cells were loaded with the Ca^{2+}-sensitive fluorescence indicator Fluo-3/AM (5 µM, Molecular-Probes, Invitrogen) in Krebs Ringer's solution at 37°C with 5% CO_2 for 30 min. The Ca^{2+} measurements were conducted at 37°C in a heat-controlled chamber (QE-1, Warner Instruments) with a confocal microscope Zeiss LSM510NLO META (Carl Zeiss) equipped with a 20x/0.8NA dipping lens (Carl Zeiss). Excitation was set at 488 nm and emission wavelengths were detected at 510 nm. The sampling frequency was set to 0.1 Hz. Carl Zeiss software (Carl Zeiss) was used to analyze the acquired images. The experiments were performed in Krebs-Ringer's buffer containing 119.0 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 20.0 mM Hepes (pH 7.4), and 11.0 mM dextrose. Drugs were bath-applied.

5-ethynyl-2´-deoxyuridine (EdU) staining and immunocytochemistry

Cells were seeded on gelatin coated 13 mm coverslips in a 24 well plate at a concentration of 20 $\times 10^3$ cells/ml. After 48 or 72 h of BCG treatment, the cells were puls-labeled with 50 µM EdU for 1-2 h and fixed with 4% PFA. The cells were blocked with PBS containing 0.3% triton and 3% BSA for 1 h at room temperature, and incubated with the primary antibody (Ki67 1:200, NeoMarkers) at 4°C overnight. Cells were then washed three times with PBS-triton and incubated with secondary antibody (Alexa 555 goat anti rabbit 1:1000, Invitrogen). EdU incorporation was subsequently detected by incubation with a reaction-mix (TRIS pH 8.8, CuSo4, Alexa-azide 488, ascorbic acid) for 30

min at room temperature protected from light. Cells were washed with PBS-triton and stained with TO-PRO3 (Molecular Probes, Invitrogen) for 5 min before mounted. The samples were evaluated with a Carl Zeiss LSM5 Exciter confocal microscope.

siRNA transfection

siRNA against PLCβ3 and PLCγ (Dharmacon) was used to reduce protein levels. T24 cells were seeded in 25 mm plates to 60% confluency and transfected with siRNA (100 µM) using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Invitrogen) according the manufacturer's instructions. Down regulation of PLCβ3 and PLCγ was confirmed by Western blot analysis.

Western blotting

After adequate treatment cells were lysed using modified RIPA buffer for 20 min at 4°C. Equal amounts of protein were separated on a 10% sodium dodecyl sulfate gel electrophoresis, followed by a transfer to a PVDF membrane. The membranes were blocked in 5% milk or BSA in TRIS-buffered saline solution plus 0.5% Tween-20 for 1 h before incubation with primary antibodies (PLCβ3, 1:1000; PLC γ, 1:1000; β-actin, 1:1000, Abcam) overnight at 4°C and further incubation with secondary antibodies (1:5000) for 1 h. Bands were detected with a chemiluminescence kit (Amersham) and visualized using Biorad.

Statistical analysis

The Ca^{2+} recording data were normalized and cells were considered responsive to a treatment if the mean fluorescence was increased by at least 50% over the baseline. All the data were presented as means ± SEM. Oneway ANOVA was used and significance was accepted at $P < 0.05$.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Legends

Figure 1| BCG elevates cytosolic Ca^{2+} in human urinary bladder cells. (**A**) Human urinary bladder cancer cells, T24, were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. Representative single cell Ca^{2+} recordings are shown in B to G. (**B**) Baseline Ca^{2+} recording of untreated cells showed no spontaneous activity. (**C**) Viability test with the purinergic receptor agonist ATP (10 μM) rapidly induced a Ca^{2+} transient. BCG stimulation provoked a Ca^{2+} response in both presence (**D**) and absence (**E**) of extracellular Ca^{2+}. (**F**) BCG induced a similar Ca^{2+} response in a different cell model, renal KU-19-20 cells. (**G**) Stimulation with PGN (250 000 units/ml) evoked an analogous Ca^{2+} response pattern as BCG did. (**H**) Statistical analyses of the different types of Ca^{2+} response to BCG in the presence and absence of extracellular Ca^{2+}. Data in the graph are presented as mean ± SEM ($n \geq 11$). Ratio F/F_0 represents fluorescence intensity over baseline.

Figure 2 | **BCG binds to target(s) that appear to locate to the ER.** Confocal microscopy images of cells incubated overnight with BCG stained with BacLight (**A**) and loaded with ER-tracker (**B**). Bright field (**D**) and merged images (**D**) show that BCG co-localizes with the ER. Lower panels show indicated zoomed area from upper panels.

Figure 3 | **The BCG-induced response involves release of Ca^{2+} from intracellular ER Ca^{2+} stores.** Cells were preincubated with indicated inhibitors and treated with BCG (arrow). (**A** and **I**) The Ca^{2+} chelator BAPTA (10 μM) significantly abolished the Ca^{2+} response. Depleting intracellular Ca^{2+} stores with the SERCA pump inhibitor CPA (50 μM) completely inhibited the Ca^{2+} response (**B** and **I**) while the $InsP_3R$ and store operated Ca^{2+} channel blocker 2APB (100 μM) entirely inhibited Ca^{2+} oscillations but had no effect on the transient response (**C** and **I**). Inhibition with the two PLC inhibitors U73122 (2-4 μM) (**D** and **I**) and ET-18-OCH_3 (2-4 μM) (**E** and **I**) completely blocked the Ca^{2+} increase. Neither the inactive U73122 analogue U73343 (2-4 μM) (**F** and **I**) nor the PI3K antagonist Wortmannin (1 μM) (**G** and **I**) had any effect, whereas the GPCR inactivator Pertussis toxin (PTX, 1 μg/ml) 6 h prior to BCG suppressed the BCG induced Ca^{2+} response (**H** and **I**). (**I**) Statistical analyses of different type of BCG-induced Ca^{2+} responses in urinary bladder cancer cells. Data in the graph are presented as mean ± SEM ($n \geq 6$). * $P < 0.05$ (Dunn's Method). All experiments were carried out in extracellular Ca^{2+} free solution.

Figure 4 | **PLCβ3 knockdown abolishes BCG-induced Ca^{2+} signaling.** (**A**) RT-PCR of the PLCβ subunit isoforms in T24 cells showed mRNA expression of PLCβ2-4 (PB1-4). Amplification of TBP and NAPDH

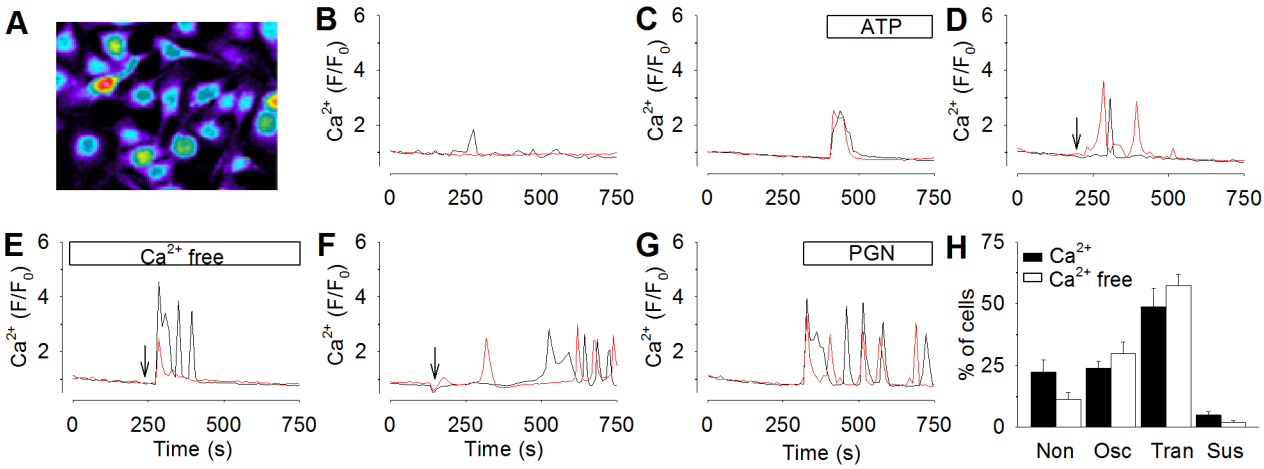
were used as controls. (**B**) Western blot analysis of PLCβ3 expression showed downregulation of protein levels after treatment with siRNA-PLCβ3 (100 nM) for 24, 36, 48 h. (**C**) The BCG-induced Ca^{2+} response was suppressed in cell transfected with siRNA-PLCβ3 for 36 h. (**D**) Statistical analyses of siRNA-PLCβ3 transfected cells treated with BCG. Data are means ± SEM ($n \geq 4$). * $P < 0.05$ (Dunn's Method). (**E**) Western blot analysis of PLCγ expression showed downregulation of protein levels after treatment with siRNA-PLCγ (100 nM) for 36 h. (**F**) The BCG-induced Ca^{2+} response was still present in cell transfected with siRNA-PLCγ for 36 h.

Figure 5 | The supernatant of BCG also stimulates Ca^{2+} signaling. Cells loaded with Fluo-3/AM were preincubated with indicated inhibitors and treated with the supernantant of BCG (sBCG, arrow). Cells showed a response to sBCG both in presence (**A** and **I**) and absence (**B** and **I**) of extracellular Ca^{2+}. The Ca^{2+} chelator BAPTA (10 μM) (**C** and **I**) and the PLC inhibitors U73122 (2-4 μM) (**D** and **I**) and ET-18-OCH_3 (2-5 μM) (**E** and **I**) all suppressed the sBCG-induced Ca^{2+} response. (**F** and **I**) The U73122 analogue U73343 had no effect on the BCG response. (**G** and **I**) Pertussis toxin (PTX, 1 μg/ml) 6 h prior to BCG reduced the Ca^{2+} response. Experiments in B to G were performed in extracellular Ca^{2+} free solution. Statistical analyses of the different types of Ca^{2+} responses induced by sBCG with and without extracellular Ca^{2+} (**H**) and with various inhibitors (**I**). Data are presented as means ± SEM ($n \geq$ 12 in H) and ($n \geq 4$ in I). *$P < 0.05$ (Dunn's Method).

Figure 6 | **BCG inhibits cell proliferation of human urinary bladder cancer cells.** (**A**) Confocal images of EdU (green) and Ki67 (red) in T24 cells treated with BCG/sBCG plus or minus promyelotic leukemia PLB-985 cells for 48 or 72 h. Nuclear staining with TO-PRO3 (blue). Treatment with sBCG alone or in combination with PLB-985 cells did not influence the expression levels of either Ki67 (**B**) or EdU (**C**). BCG alone or in combination with PLB-985 cells had no effect on Ki67 (**D**) whereas BCG significantly decreased EdU expression independently of PLB-985 (**E**). Data are means ± SEM ($n = 3$). * $P < 0.05$ (Holm-Sidak method).

Figure 7 | **PLC critically regulates BCG-controlled cell proliferation.** (**A**) Confocal images of EdU (green) stained cells treated with BCG plus or minus U73122 or U73343 for 72 h. Nuclear staining with TO-PRO3 (blue). Treatment with BCG plus or minus U73122 or U73343 had no effect on the Ki67 expression (**B**) whereas treatment with U73122 abolished BCG's effect on EdU expression (**C**). Data are means ± SEM ($n = 8$). * $P < 0.001$ (Holm-Sidak method).

Figure 1. Karlsson *et al.*



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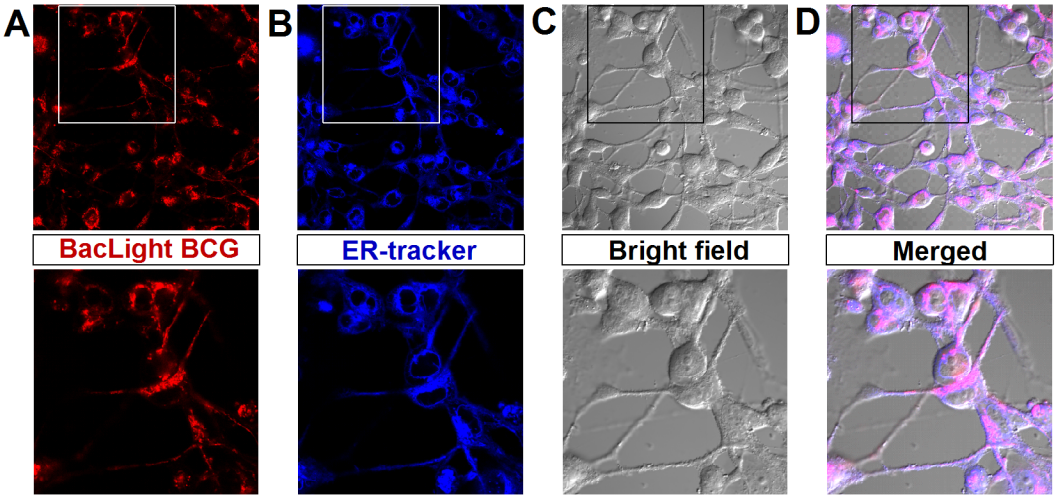
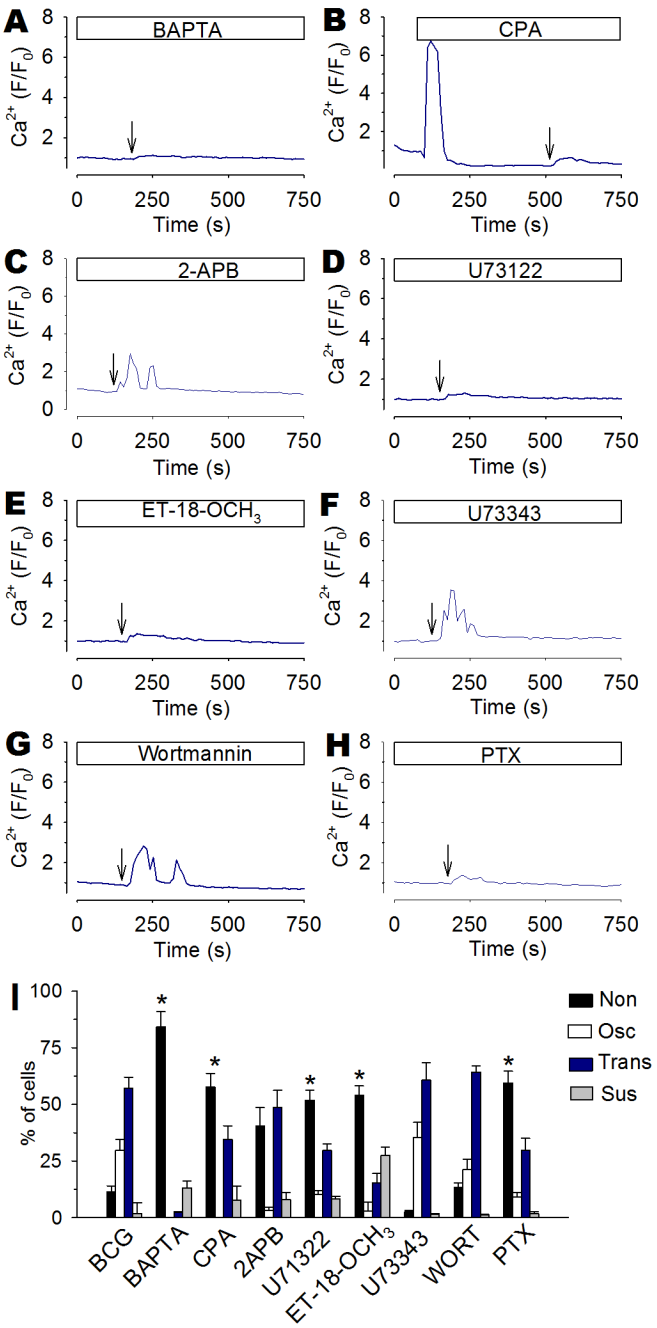


Figure 3 - Karlsson *et al.*



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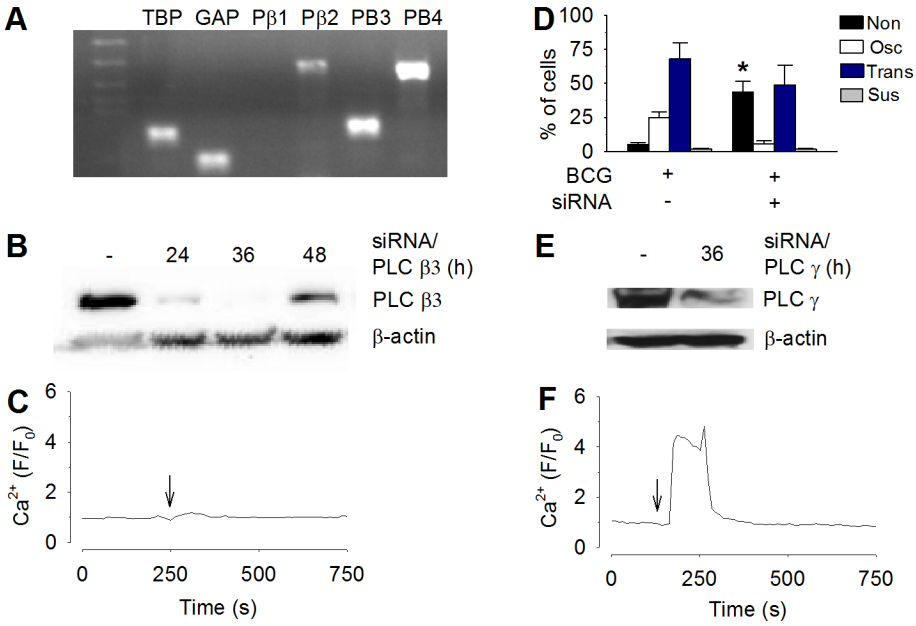

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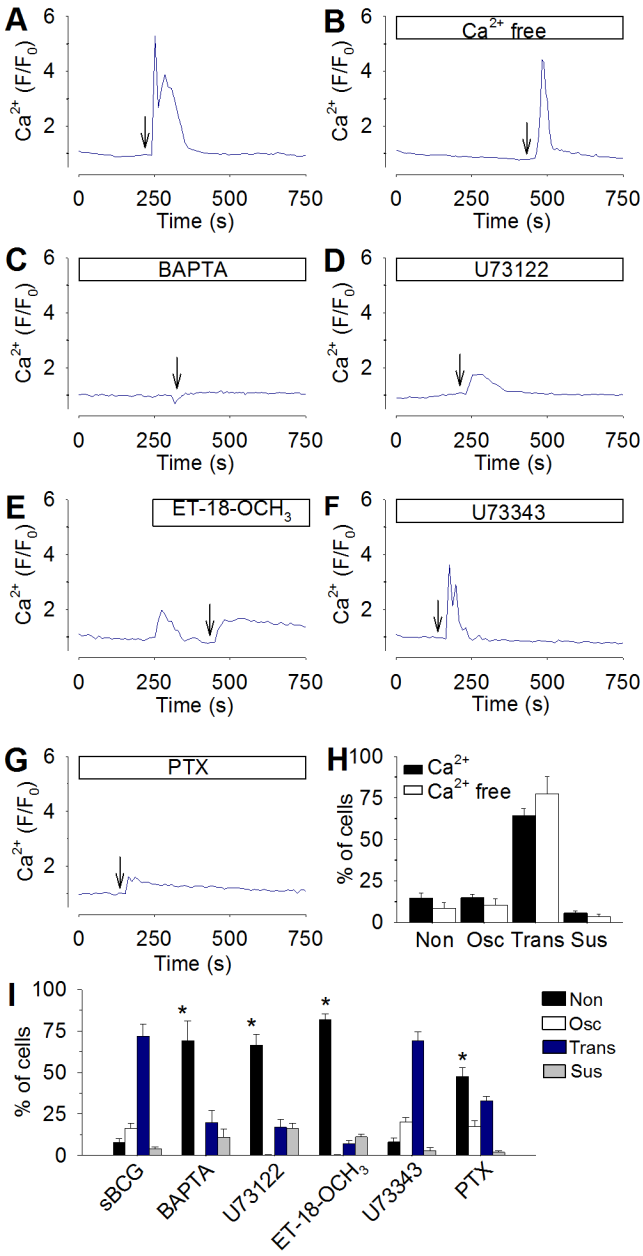
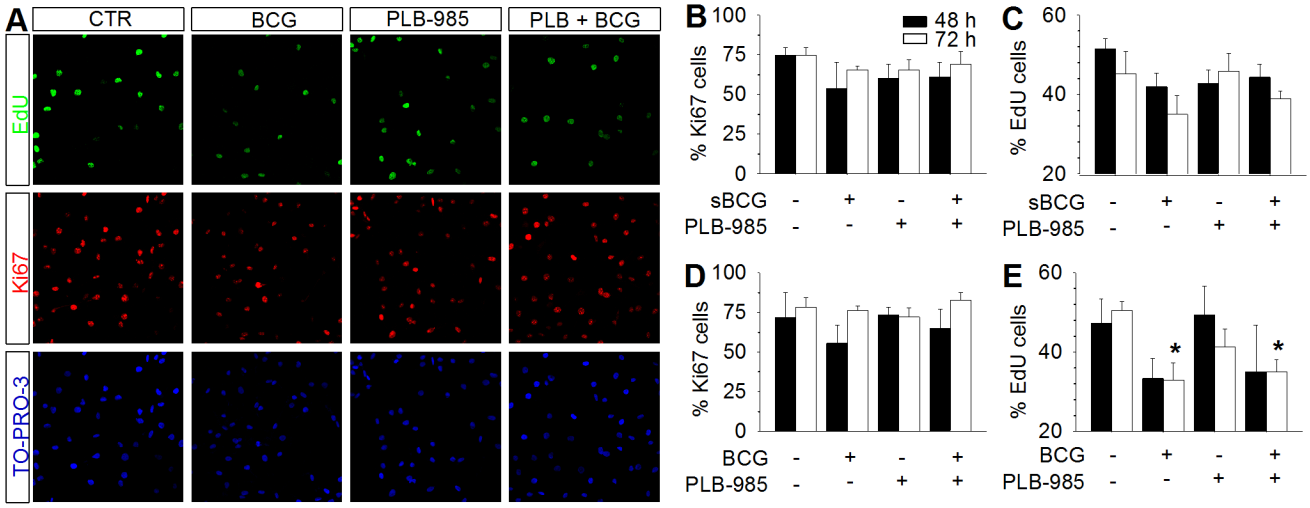


Figure 6. Karlsson *et al.*



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