BK channels in human glioma cells

Dissertation

zur Erlangung des akademischen Grades
doctor medicinae (Dr. med.)

vorgelegt dem Rat der Medizinischen Fakultät
der Friedrich-Schiller-Universität Jena

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geboren am 14. Februar 1973 in Singen (Hohentwiel)

FIGURES

ABBREVIATIONS

4-AP	4 - Aminopyridine
BK channels	Big conductance calcium activated potassium channels
Ca^{2+}	Calcium
$[Ca^{2+}]$	Calcium concentration
$[Ca^{2+}]_i$	Calcium concentration in the cell interior
CHTX	Charybdotoxin
Cl^-	Chloride ion
CLTX	Chlorotoxin
CNS	Central nervous system
CS^{2+}	Cesium ion
DNA	Desoxyribo - Nuclein - Acid
EGF	Epidermal growth factor
EGFR	EGF receptor
ET-1	Endothelin-1
FCS	Fetal calf serum
G	Conductance
G/Gmax	Normalized conductances
G_{max}	Peak conductance
hbr	mRNA family that encodes for BK channels in the human brain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF	High power field (40x)
hslo	Human variant of the Slowpoke gene
HUVEC	Human vascular endothelial cells
IBTX	Iberotoxin or Iberiotoxin
IC_{50}	50% inhibitory concentration of a blocker
IK channels	Intermediate conductance potassium channels
IK_{Ca}	Calcium activated potassium currents
K^+	Potassium ion
$[K^+]$	Concentration of potassium
$[K^+]_e$	Extracellular concentration of potassium
K_{ATP} channels	ATP sensitive potassium channels
K_{Ca} channels	Calcium activated potassium channels
Kd	Delayed rectifying potassium channels
K_{ir} currents	Inwardly rectifying potassium channels
K_v1.3 channels	Voltage gated potassium channels of the 1.3 type
MDCK	Madin-Darby canine kidney cells
MEM	Modified Essential Medium
mM	Millimolar

mslo	Mammalian variant of the Slowpoke gene
Na^+	Sodium ion
NGF	Nerve growth factor
NPPB	5-Nitro-2-(3-phenylpropylamino)benzoic acid
NSCC	Non selective cation channels
PBS	Phosphate buffered saline
PDGF	Platlet derived growth factor
pF	Pico Farad
PKA	Protein kinase A
PKC	Protein kinase C
P_o	Open probability
pS	Pico Siemens
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
SEM	Rtandard deviation of means
SK channels	Small conductance
slo	Slowpoke gene
TEA	Tetraethylammonium
$V_{½}$	Voltage of half maximal channel activation
VEGF	Vascular endothelial growth factor
VLG_{Ca}	Voltage gated calcium channels
V_m	Clamped membrane potential
V_{rev}	Reversal potential
WHO	World Health Organization

1 Introduction

Human glioma cells express a variety of voltage-gated ion channels including Na^+ and K^+ channels. Different types of K^+ currents have been reported, namely voltage-gated K^+ currents [1], inwardly rectifying K+ currents [2] and large-conductance K_{Ca} channels [1,3]. BK channel currents were frequently observed suggesting an ubiquitous expression of these channels in cultured glioma cells [4]. Despite their stable expression in glioma cells a functional role of BK channels has not been identified. Here we addressed the question whether BK channels are involved in the migration and proliferation of the human astrocytoma cell line 1321N1. This study deals with the question whether a *specific channel* plays a role for the pathobiology of human brain tumors

1.1 Pathology and epidemiology of gliomas

Tumors of glial origin are the most common primary brain tumors and make up more than 40 % of all CNS neoplasms. They occur with two incidence peaks, one in childhood and one between the fourth and fifth decade. Glioblastoma also occurs between the 6^{th} and 7^{th} decade.

The WHO has proposed a classification system which distinguishes four different histologic subforms [5]. A higher grade in this classification system signifies more mitoses, more vascular proliferations, giant cells and areas of necrosis. Strikingly even the histologically relatively well-differentiated grade II astrocytomas show a marked tendency to diffusely infiltrate the brain. In other words even a tumor, which appears well differentiated under the microscope, can be biologically highly malignant due to the tumor cells capacity to actively migrate along structures within the neuropil. It is not a rare event to find glioma cells not only in the infiltration zone near the tumor nucleus but also in areas very distant from the tumor center. Surgical resection will therefore always leave tumor cells behind. To make matters worse, the remaining tumor after surgery shows the inherent tendency to progress to a more malignant phenotype. All these phenomena, the migratory potential, the diffuse extension and the potency of malignant progression, account for the low survival rates even after all therapeutic modalities have been utilized

(5yr postsurgigal survival: 10 – 50 % for astrocytoma grade II versus almost 0 % for glioblastoma multiforme) [6].

1.2 Focus of glioma research

In the past, much effort has been shown in the investigation of molecular biological events occurring at the various stages of glioma progression. Many relevant underlying genetic alterations have been identified. For instance, tumor suppressor gene p53 inactivation has been found to be an early genetic event of initial glioma generation. Other chromosomal changes do not correlate with tumor initiation but are related to glioma progression, malignant transformation, and higher recurrency rates.
Namely the passage from a well-differentiated picture to anaplastic astrocytoma is marked by allelic losses of chromosome 9p, 13q and 19q.
Recently on the other hand a molecular benefit marker could be identified. Loss of heterozygosity of chromosome 19q and 1p in oligodendroglial tumors are correlated with sensitivity for chemotherapy and longer recurrency-free intervals [7]. To date, however, it is not clear whether this marker does also mean better prognosis for the patients. Its possible relevance for astrocytic tumors is under current investigation. The putative tumor suppressor genes and the genes responsible for chemotherapeutic sensitivity are not yet well characterized.

Autocrine and paracrine signals from the microenvironment of the tumor are translated into a cellular response by cell – surface receptors. A role in the initiation and progression of neoplasia was attributed to members of the tyrosin kinase receptor family not only in experimental models of neoplasia but also in human cancer. Many investigations have dealt with the role of overamplification of growth factor genes and their receptors which can be frequently observed in glioma : The platelet derived growth factor (PDGF) and the epidermal growth factor (EGF) are the two best studied. The appearance of EGF receptor overamplification is a step in tumor progression from anaplastic astrocytoma to glioblastoma [8, 9]. About 40 % of glioblastomas display EGF receptor overamplification [10, 11]. The level of overexpression correlates with the tumor grade. PDGF gene and receptor have been also found in many glioma cell lines as well as in many surgical specimen [12-14].

There is much hope that further molecular biological investigations will achieve identification of genetic markers that are still more closely linked to prognosis or other important factors.

Apart from this very promising molecular genetic research on glial tumors focus has also been laid on cell biological aspects. These include among others the investigation of distinct steps of the cell signalling cascade and alterations in the cell membrane properties. In the latter respect, ion channels are very central to be studied. Since the patch-clamp method is available, voltage-gated ion channels have been studied intensively. These studies have the great advantage over molecular biological and histologic investigations that they allow a functional characterization of a single protein in real time under relatively physiological conditions. Thus they provide insights into the dynamic electrical activity of the cell membrane and how the cell responds to environmental stimuli that influence the cells membrane properties. Much can be deduced from those types of studies.

1.2.1 Voltage-gated ion channels in human gliomas

It became clear in recent years that the glial cell is not a passive element of the brain but a very active one [15]. As one reflection of their active role in the brain glial cells are outfitted with a variety of voltage-gated ion channels, which they use to buffer the ion concentration of the extracellular fluid. Another key finding is that glial cells have a spectrum of growth factor receptors [16] through which mitogenes can stimulate the cells. Throughout life glial cells can reenter the proliferative cycle and replace dead tissue [17]. The proliferative zone and source for this replacement of cells is the subependymal layer from where the cells migrate to the area of injury. This regeneration of dead tissue by glial cells has been termed reactive gliosis. Naturally cells with such a high proliferative capacity are also occasionally prone to malignant degeneration.

In more recent times, voltage-gated ion channels were studied in glioma cell lines or primary glioma cultures and, in few laboratories, also in cells from living human tumor tissue slices. The first reports of voltage-gated ion channels in human astrocytomas were published in the late 80s. Brismar and Collins described six astrocytoma cell

lines in which four different channel types were present: Inward rectifying K^+ channels, large conductance K_{Ca} (BK) channels, delayed rectifier K^+ channels and voltage-gated Na^+ channels [1, 2].

Na^+ channels

In tissue slices and early primary cultures of brain tumors several investigators found high Na^+ channel densities [18-21]. In some cells their density was so high that the cells even displayed the ability to generate action potentials. These findings came as a surprise because before that only cell lines had been studied and cultured cells displayed only a small fraction of the Na^+ currents seen in slices. For some unknown reason glioma cells lost or downregulated their sodium channels during culture passage. Recently we were able to show that NGF can induce Na^+ channels via autocrine loops among tumor cells and thus provide a model of how Na^+ currents are regulated [22]. The present hypothesis is that high Na^+ channel densities seen in tumor cells in vivo are maintained by constant stimulation from NGF in the microenvironment. It is still controversial whether such tumor cells play a role in tumor-associated epilepsy [23]. Moreover it is unclear which role Na^+ channels play in the malignant behaviour of the tumor. We will not further discuss Na^+ channels in this work since they have already been topic of our recent publication [22].

K^+ channels

While Na^+ channels in excitable cells are responsible for action potential generation voltage-gated K^+ channels are key molecules for maintainance of the resting membrane potential and for repolarisation following the action potential. Following membrane depolarization K^+ permeability increases as voltage-gated K^+ channels open and thus repolarisation is achieved.

In nonexcitable cells their role to date has not been conclusively determined. Like in excitable cells K^+ channels have been found to stabilize the membrane potential following depolarizing events. Moreover voltage-gated K^+ channels are thought to be involved in volume regulatory mechanisms [24]. These channel-dependent cell functions are important in proliferation and migration of cells.

In the following we first will give a short overview on the current knowledge and some hypotheses regarding the role of K^+ channels in cell proliferation and secondly comment on their putative role in cell migration.

1.2.2 Voltage-gated K^+ channels and cell proliferation

Three lines of evidence point to an essential role for K^+ channels for the progression of cells through the G1 phase of the cell cycle, i.e., (i) K^+ channel blockers inhibit mitogenesis, (ii) mitogens increase K^+ channel activity or expression, and (iii) K^+ -channel openers stimulate cell proliferation.
Evidence for the involvement of K^+ channels in proliferation now exists for a large variety of cell types. There is now also some idea of how channel block might influence proliferation – at least two hypotheses have been put forward (for review see: [25]).

The first major theory assumes that K^+ channels sustain and fine–tune the **membrane potential changes** necessary for proliferative activity. Tumor cells were shown to be generally strongly depolarized compared to nonmalignant cells [26]. In order to enter the cell cycle cells have to take a hyperpolarizing step that leads to Ca^{2+} influx. An increase of $[Ca^{2+}]_i$ is the trigger for the transition from G1 to S phase during mitosis. An electrical driving force sustains Ca^{2+} influx across a hyperpolarized cell membrane. It has been shown that K^+ channels sustain this electrochemical driving force. The pathway for the Ca^{2+} entry has not yet been elucidated but in C6 glioma cells it was observed that it is a non selective cation channel (NSCC)[27]. A hyperpolarized membrane potential is also a prerequisite for IP_3 production and thus intracellular Ca^{2+} release [28]. Similar observations to those in melanoma cells were made in a study on colon cancer cells where K^+ channel inhibitors blocked Ca^{2+} entry and proliferation. In that study K^+-ATP channels sustained the electrochemical driving force for Ca^{2+} [29].
A second mechanism that might require membrane hyperpolarisation as its driving force, could be the Na^+-dependent transport of metabolic substrates and ions into the cells [30].

Rouzaire-Dubois et al. [31, 32] have proposed an alternative theory. According to these authors interference with a **volume regulatory mechanism** is responsible for the inhibition of proliferation by K^+ channel antagonists. Their study of C6 glioma cells found an inverse relationship between the rate of proliferation and cell volume.

Increasing the cell volume and abolishing regulatory volume decrease by the application of broad-spectrum channel blockers (TEA, NPPB and CS^{2+}) or hypertonic media significantly slowed the proliferation rate of the cells. Two hypotheses were put forward to explain the effect: Either cell swelling may decrease the concentration of cell-regulating proteins or alternatively lead to cytoskeleton rearrangements that might interfere with a multitude of cell functions [31, 32].

There is now evidence that K^+ channel blockers inhibit proliferation by arresting proliferating cells in the G1 phase of the cell cycle rather than by a nonspecific slowing of the cell cycle. This has been established by flow cytometric studies of several specific markers of the G1 phase in MCF-7 human breast cancer cells [33] [34], in the GH3 pituitary cell line [35] and in spinal cord astrocytes [36]. For an extensive review also see Wonderlin and Strobl [25]. It was postulated that blocking K^+ channels inhibits a Ca^{2+} influx, which is crucial for a Ca^{2+} and calmodulin dependent phosphorylation of the cell cycle protein pp34. This protein is necessary for the cell cycle progression through the so-called START restriction point between the G1 and S phase [37].

The most convincing studies **linking a specific channel to proliferation** are the ones that have used the high affinity blockers to inhibit proliferation. High affinity toxins have the advantage that they do not interact with other targets than the specific channels they are designed for and do not enter cells [38-40]. A definite link between a specific channel type and G1 progression so far has been established for the $K_v1.3$ channels by using the specific blocker MGTX [38]. Recently there have also been clues that K_{ATP} channels might play a role [33, 41]. Three studies so far have demonstrated that a specific block of BK channels led to an inhibition of proliferation. [35, 42, 43]. What is intriguing about the involvement of K_{ATP} and K_{Ca} channels is that they are gated by intracellular ligands thus linking intracellular metabolic processes with membrane properties. In BK channels, as we shall discuss in further detail later, might constitute such a link between membrane potential and Ca^{2+} signalling since they are gated by intracellular Ca^{2+} [44].

K^+ channel blockers inhibit mitogenesis but on the other hand K^+ channel openers stimulate cell growth. It was observed that minoxidil potentiates the mitogenic effects

of fetal calf serum in vitro on NIH 3T3 fibroblasts by opening K^+ channels and is also able to potentiate the mitogenic effects of the growth factors platelet-derived growth factor and insulin-like growth factor 1 [45]. Moreover acetylcholin an activator of BK channels induced proliferation of human glioma cell lines [46].

Taken together these studies constitute strong evidence for a crucial role of K^+ channels in proliferation. A completely nonspecific effect of these various K^+ channel modulators is very unlikely due to the fact that their chemical structures differ greatly from each other.

Glioma cells

The role of K^+ channels in **cell proliferation of gliomas** was first investigated in a human astrocytoma cell line [47]. The authors of this study demonstrated that K^+ channel inhibitors were effective inhibitors of brain tumor cell growth by interfering with the intracellular Ca^{2+} signalling mechanisms through membrane depolarisation.
Chin et al. presented more evidence that K+ channels play a role in the proliferative activity of malignant astrocytoma cell lines in 1997 [48]. The authors found two types of K^+ currents in the two cell lines they studied: BK channels and a 4-AP sensitive outward rectifier current. K+ channel blockers could inhibit proliferation but only the nonspecific agents 4-AP and TEA were used. From these studies it is unclear if specific channels are essential for growth.

Indirect evidence that BK channels play a role in proliferation of glioma cells comes from studies done on **reactively proliferating astrocytes**. While large inwardly rectifying currents are a marker of differentiated glial cells, proliferating cells downregulate inward rectifying K^+ currents and upregulate outward rectifying K^+ currents. The high level of K_{ir} currents in differentiated glial cells guaranties a stable hyperpolarized membrane potential while proliferating glial cells have a less stabile, more depolarized membrane potential [36, 49, 50]. More precicely G1 arrested glial cells displayed increased outward rectifying K^+ currents [36]. In one study it was observed that these increased outward rectifying K^+ currents were mediated by BK channels linking proliferative activity in glial cells to one specific type of ion channel [49]. In addition IBTX, a specific BK channel blocker, blocked proliferation of reactive

retinal astrocytes however only when the membrane was depolarized with 15 mM $[K^+]_e$ [43, 51]. The exact functional aspects of K^+ channel activity remain unresolved. However the available data suggest a role of BK channels for growth factor stimulated proliferation such as elevated $[K^+]_e$ which may occur in areas of tumor necrosis or ischemic regions where $[K^+]_e$ can reach values of up to 135 mM compared to the normal 5 mM. Raising the $[K^+]_e$ depolarizes the cell membrane and consequently increases the activity of BK channels [5]. Earlier, moderately elevated $[K^+]_e$ was shown to be linked to BK channel activity and DNA synthesis of normal glial cells [43]. Here, we tested the influence of an elevated $[K^+]_e$ on the proliferation of the astrocytoma cell line 1321N1.

In summary, it is conceivable that BK channels are involved also in glioma proliferation. Since they are ubiquitously present in glioma cell lines it is intriguing to characterize their physiological properties and study their function with the specific blocker IBTX.

1.2.3 Voltage-gated K^+ channels and cell migration

Data on the physiology of migration have been gathered from a vast array of different cell types. The key players, which bring about cell propagation are the cytoskeleton, surface receptors for extracellular matrix proteins and ion channels. In the migrating cell these components interplay in a complex and still poorly understood manner **(Figure 1)**. Migrating cells are polarized and have a flat leading end and a rounded retracting end. What has so far been established is that actin is depolimerized at the rear of the cells; the actin framents are then transported to the leading edge where they are again polimerized. The enzymes involved in actin filament depolymerisation are Ca^{2+} dependent [52] [53]. Thus at the rear pole of the cell actin depolimerisation requires Ca^{2+} -oszillations and at the same time RVD (regulatory volume decrease) is brought about by cyclic activity of K_{Ca} channels (for review see: [24]). The interplay between ion channels, cell volume and actin polimerisation was experimentally disclosed in MDCK cells (Madin-Darby canine kidney cells). Schwab and Oberleithner showed that about 20 % of the cellular K^+ leave MDCK-F canine kidney cells during oscillating K_{Ca} channels bursts. K^+ is accompanied by Cl^- and HCO_3 and water leading to a shrinking of the cells [53-55]. Moreover cell swelling as well as the

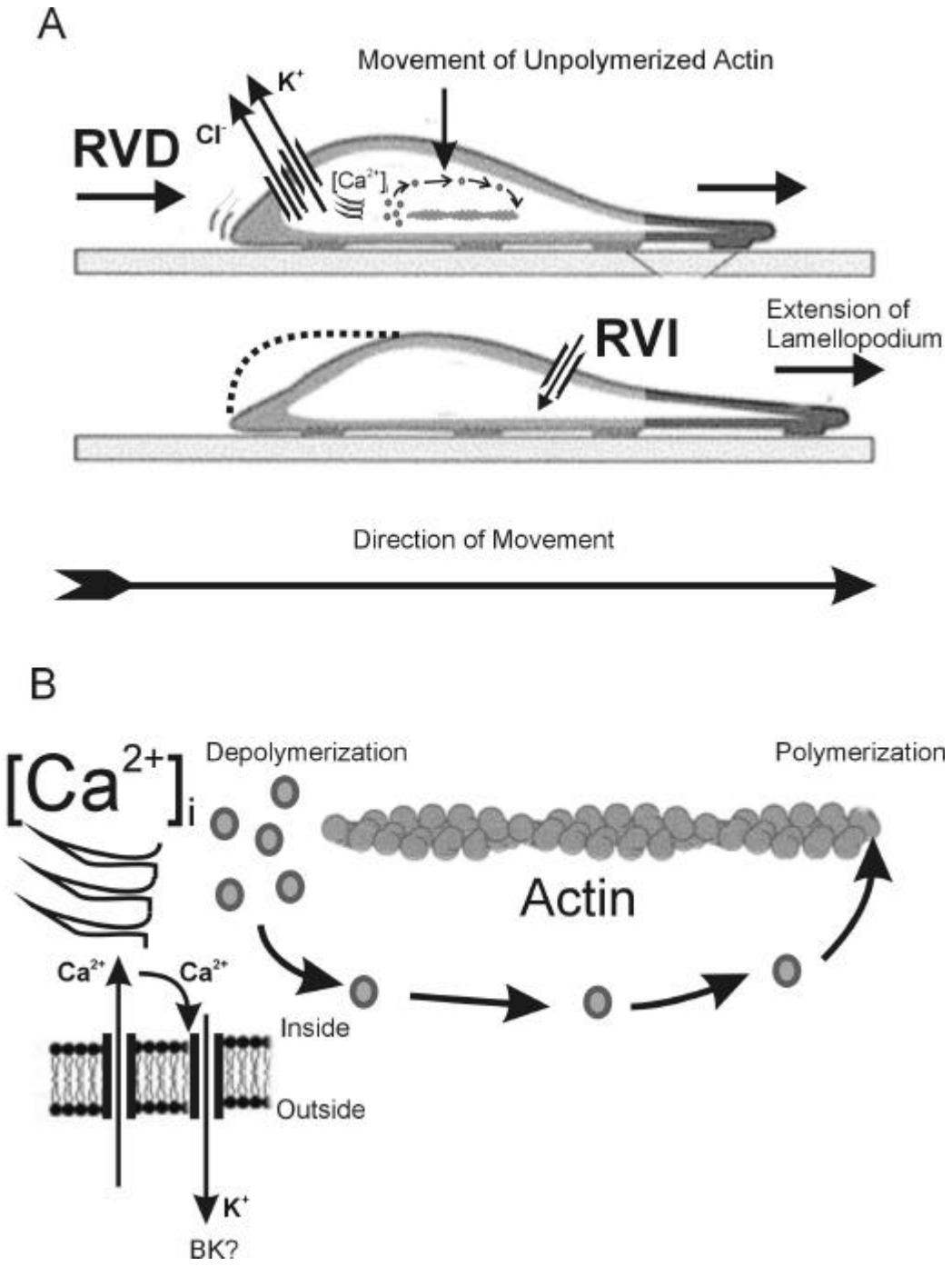

Figure 1 – Interplay of ion channels and the cytoskeleton in cell locomotion.
A) Ca^{2+} activated K^+ channels in migrating cells are functionally polarized towards the rear. Their activation causes the rear end of the cell to shrink and retract because water is expelled together with K^+ (RVD – regulatory volume decrease). This in turn causes a depolymerization of the actin cytoskeleton (see B). At the front pole the lammelopodium can extend because actin filaments are reassembled there (see B). Moreover the influx of ions through largely unknown pathways causes this region to swell thus propulsing the cell body (RVI – regulatory volume increase).
B) Mechanism of actin turnover in the cell interior of a migrating cell. Actin depolimerization at the rear end of the cell requires oscillations of $[Ca^{2+}]_i$ because it involves Ca^{2+} dependent enzymes. These oscillations of $[Ca^{2+}]_i$ have been experimentally detected only at the rear end of the migrating cells and were shown to be driven by a cyclic activity of Ca^{2+} activated K^+ channels.
Hypothetically BK channels could play a significant role in both RVD (regulatory volume decrease) and in eliciting the calcium oscillations, which cause actin depolimerization.
[Modified according to [24 and [53].

inhibition of RVD with the K^+ channel blocker CHTX induces actin depolimerisation [55]. In the extending processes where actin is polymerized Na^+/H^+ exchangers and nonselective cation channels are activated and lead to a regulatory volume increase which again induces regulatory actin disassembly [24]. After the cell has thus retracted its rear end and extended the front pole the chain of events is repeated. This complex mechanics requires asymmetric distribution of channels and it was indeed shown by Reinhardt et al. (1998) that K^+ channels are functionally polarized towards the rear end in migrating cells [56]. Likewise the Ca^{2+} spiking activity is polarized being highest at the rear where actin polimerization depends on it.

Glioma cells

There is now a large fund of data on the biological properties of invasive cells, which supports the multistep process which tumor invasion of healthy tissue is. Moleculargenetic investigations have made out that the events during invasion are similar to processes in the developing tissues. During malignant alteration cells therefore aquire a fetal genetic profile. In general invasive behaviour is constituded by alterations of specific cell properties that are distinct from the general pattern of genetic alterations in non-migrating malignant cells. Genes that have been associated specifically with invasion in melanoma cells and glioma cells are 18A2/mts1, nm 23 and very recently p311 [57-61]. For a review of the multitude of characteristic genetic alterations, which have also been identified in invasive glioma, cells see: [62, 63].

Invasive behaviour generally requires two capacities from the tumor cells, namely cell motility and the ability to modify the environment. Mechanisms that go along with glioma cell invasiveness which have been identified so far are alterations in cell adhesion molecules [64, 65]), extracellular matrix proteins and their surface receptors [64-66] and secretion of proteolytic enzymes [67-69]. Primary brain tumors tend to invade along existing anatomical structures in the brain. They diffusely spread into the white matter in the hemisphere along nerve fibers, blood vessels, and the Virchow Robin spaces and between pial and arachnoid spaces [66]. The gaps they have to permeate are very narrow (20 nm) and as a consequence cells have to undergo a profound volume decrease. Little attention has been paid to the adaptative mechanisms that enable glioma cells to reduce their volume. For normal astrocytes it

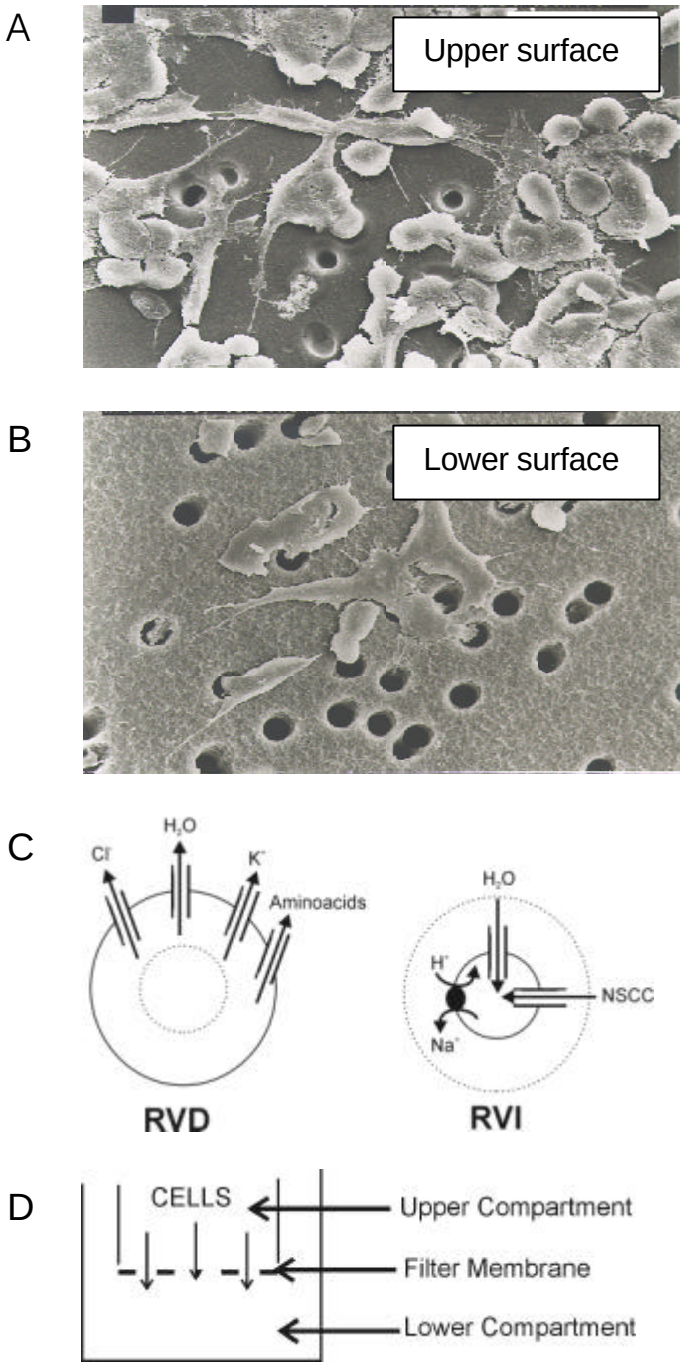

Figure 2 – Transwell assay to study cell migration

A) Transwell assay to study cell migration through 8µm pores. Migration occurs from high cell density in the upper compartment towards a low cell density in the lower compartment of the well through small pores. For cell counting the upper surface is cleared of cells with a cotton swab. The cells depicted here are SH-SY5Y neuroblastoma cells photographed with an electron microscope.

B) Cells that have migrated through the pores adhere to the lower surface of the membrane.

C) Putative mechanisms for cell volume regulation following experiments with osmotic stress (see [24]. RVD (regulatory volume decrease) is thought to be caused by Cl^-efflux, K^+ efflux or alternatively by aminoacid efflux. RVI (regulatory volume increase) is thought to follow activation of the Na^+/H^+ exchanger or of NSCC (non selective cation channels).

K^+ channel activators interfere with cell migration by permanently shrinking the cell, whereas K^+ channel blockers influences cell migration through permanent cell swelling thus inhibiting the adaptative volume changes during cell locomotion (see Figure 1).

D) Schematic of a Transwell assay (Boyden Chamber).

was shown that RVD (regulatory volume decrease) is mediated via K^+, Cl^-, amino acid and polyol efflux, K^+ permeability being the rate limiting step [70, 71]. Likewise in glioma cells the regulatory volume decrease is probably a consequence of mainly K^+ and Cl^- efflux through ion channels. To date however only few studies exist on the role of ion channels in glioma migration. Soroceanu et al. (1998) [72] recently proposed that Cl^- channels are upregulated in glioma cells and chloride efflux brings about cell shape changes. Indeed several ion channel blockers inhibited transwell permeation in that study. The authors concluded that chloride channels were key players in RVD of glioma cells because they used CLTX assuming it to be a high affinity chloride channel blocker. Unfortunately all the other blockers tested were relatively unspecific. Recently however CLTX was found not to block chloride channels [73]. The theory that voltage gated chloride channels alone are responsible for volume regulatory mechanisms is therefore questionable. In fact all the blockers used in their study also block K^+ channels, namely of the BK type. It might therefore well be that BK channels are the actual key players in transwell permeation of glioma cells **(Figure 2)**.

Another study on glioma cell migration strongly implicates a role for BK channels as their permanent activation with acetylcholine effectively inhibited migration [46]. This is in accordance with the work of Schwab et al. (1999) [55] who could inhibit MDCK cell migration by both permanent activation and permanent inhibition of K_{Ca} channels. They conclude that migration requires cyclic channel activity [55]. However the study did not investigate the effects of IBTX, which specifically blocks BK channels.

Because BK channels can be activated by Ca^{2+} oscillations and rearrangements of the cytoskeleton and because they conduct large K^+ currents when they are activated, they hypothetically are ideal candidates for fast, effective adaptative volume changes during cell migration. No study so far has directly investigated the role of BK channels with a specific blocker in glioma migration so this became the second focus of the present study.

1.3 BK channels – structure, pharmacology and regulation

K_{Ca} channels can be grouped into three categories: Small conductance (SK), intermediate conductance (IK) and big conductance (BK) types. These differ not only in their œonductance values but also in voltage dependence, Ca^{2+} sensitivity and pharmacology. The small (SK) and intermediate (IK) conductance K_{Ca} channels are not voltage sensitive and are activated by submicromolar [Ca^{2+}] which means they are both much more Ca^{2+} sensitive than BK channels. BK channels are completely blocked by 1 mM TEA SK and IK channels are relatively insensitive to the drug. SK channels are sensitive to apamin. IK channels are blocked by CHTX and clotrimazole.

BK channels are found in neurons where they control the action potential waveform and the cell excitability. Neonexcitable cells where their function is less clear also express them.
Notably the gene coding for BK channels is a single gene located on chromosome 10 at band 22.3. Chromosome 10 rearrangements are very common in glioblastoma [74, 75].

The channels are composed of four α - β heterodimers each consisting of an α and β subunit [76, 77] **(Figure 3)**. The larger α subunit is homologous to the pore forming subunits of other K^+ channels [78]. The smaller β subunit shows no homology with other ion channel subunits [79].
The isolated α subunit has ion conduction properties but coupling to the β subunit has dramatic effects on voltage- and Ca^{2+} -dependent gating of the channel structure in that it shifts the current voltage curve to the left and increases Ca^{2+} sensitivity [80]. The isolated β subunit does not have ion conduction properties.
The average single channel conductance in symmetrical [K^+] (140 mM) ranges from 150 to 650 pS. There is a high selectivity for K^+ ions, and the channels are essentially impermeable to cesium ions.

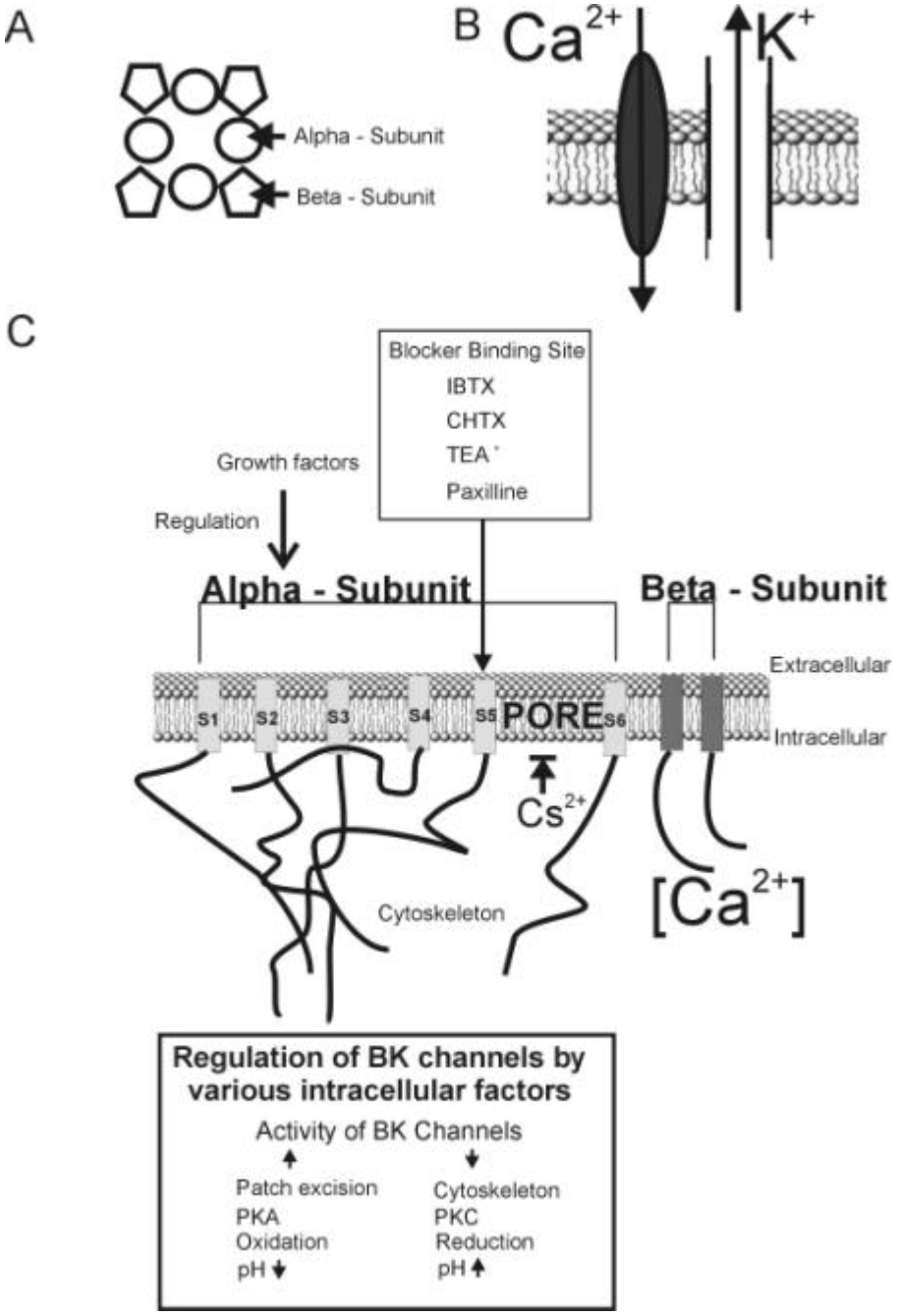

Figure 3 – Structure, pharmacology and regulation of BK channels.
A) BK channels consist of 4 heterodimers each composed of an α- and a β-subunit. The four α -subunits consist of six transmembrane segments, form the channel pore and conduct K^+ ions alone. The β-subunit accounts for the channels Ca^{2+} sensitivity (modified according to [76]).
B) BK channels may be functionally colocalized with voltage–gated Ca^{2+} channels, which may provide the Ca^{2+} peaks required for BK channel gating [132].
C) BK channels are regulated by many factors including growth factors, the PKA (protein kinase A), PKC (protein kinase C), oxidation and the intracellular pH. They are also regulated by the actin cytoskeleton. The channels are highly selective for K^+ ions and are impermeable for CsCl. The binding site for blockers is at the S5 – S6 linker rather far away from the voltage sensor in the pore [124].

Since BK channels are both voltage and ligand gated the question arises whether or not only one of these is sufficient for channel activation. It was suggested that voltage is the prime activator because it was observed that at nanomolar [Ca^{2+}] strong depolarizations can activate BK channels and they can even open in response to depolarization without the requirement of Ca^{2+} [81-83]. This raises the question whether in resting cells BK channels are at all active around the resting membrane potential.

Besides by membrane depolarisation however they can be activated by many other conditions – both intra– and extracellular stimuli. Activation can also occur by all external factors that elevate $[Ca^{2+}]_i$ such as many growth factors, by channel protein phosphorylation, interaction with the cytoskeleton, high intracellular pH and oxidative reactions [4, 44, 80, 84-93]. As it was shown for instance for human endothelial cells, growth factors strongly stimulate BK channel activity [94]. At nanomolar Ca^{2+} levels these channels were also activated after stimulation with bradykinin or acetylcholine via an increase of $[Ca^{2+}]_i$ [46]. In vivo such stimulatory mediators are very likely to be present in the environment of the glioma cells, because tumor cells have been shown to secrete neurotransmitters and different growth factors [10]. In gliomas, one of the most important and best-studied growth factors is the epidermal growth factor (EGF). The following chapter gives a short overview on EGF and links it to proliferation and migration of glioma cells and to BK channels. That all these environmental and intracellular factors can modulate BK channels suggests that when several factors act in concert *in vivo* significant channel activity might also be found at typical resting membrane potentials in nonexitable cells, eg., cells that are not depolarized to very positive potentials during an action potential. It was one of the aims of this study to investigate, whether significant BK channel activity does occur around typical resting membrane potentials in 1321N1 human astrocytoma cells.

1.4 Epidermal growth factor (EGF) in gliomas – role for cell proliferation, cell motility and activation of BK channels

The gene for the EGF receptor (EGFR) has been mapped to chromosome 7 and was named erbB1 [95]. Its overamplification occurs during the transition from astrocytoma

to glioblastoma and can be found in 40 % of all cases of glioblastoma. EGF reacts only with this receptor. EGFR is autophosphorylated in response to coupling with the ligand thus activating a cascade of other intracellular events [96].
Glioma cells can synthesise EGF and the cells crosstalk in autocrine loops. Proliferation could be inhibited by the disruption of such loops with antisense against the receptor DNA [12, 97, 98].
The human astrocytoma cell line 1321N1, which was used here, is known to have a high density of EGF receptors but it has not been studied whether receptor stimulation has any effect on proliferation or migration in these cells. Investigation of other glioma cell lines shows that in some EGF is a potent mitogen and in some a potent motogen. It is unknown in which context EGF stimulates proliferation and in which context it enhances cell propagation and also the exact cell biological mechanisms following receptor stimulation remain unresolved.

Chen et al. [99] demonstrate that EGF stimulates **cell motility and proliferation** through separate signalling pathways. Kinase negative receptor mutants elicited cell movement whereas kinase positive receptor mutants stimulated proliferation [99]. To date details of the events that lead to enhanced proliferation on the one hand and enhanced motility on the other hand remain obscure (for a broad review see: [100]). There is general understanding that cells cannot proliferate and migrate at the same time ('go *or* grow'). Cell temporarily arrest in cell cycle to migrate and only after having migrated do they reenter the cell cycle [101].

Proliferation

Recent research hints that the mechanism by which EGF stimulates **cell proliferation** might be a modulation of the membrane potential. Pandiella et al. (1989) [102] demonstrate that the effect of acute EGF application to cells overexpressing EGFR was a rapid hyperpolarisation followed by persistent swings of the membrane potential. The authors identified K_{Ca} channels as mediators of the EGF action [102]. It is known that membrane hyperpolarization is required for progression through the G1 phase of the cell cycle. The involvement of BK channels in the cells response to EGF action described for fibroblasts and lymphocytes could also be the trigger mechanism for astrocytoma cell growth following mitogenic stimulation with EGF. If this is the case then it should also be possible to inhibit

growth factor induced proliferation with specific blockers of BK channels. Interestingly, in endothelial cells growth factor-induced proliferation was shown to be blocked by the specific BK channel blocker IBTX [42]. The same was observed in Muller glial cells for EGF induced proliferation [43]. In summary, there is ample evidence that during the chain of events following mitogen stimulation BK channels are upregulated and crucial for proliferation. However, no details about the mechanism are discovered yet and it has not been determined in which contest and in which cells growth factor stimulation activates BK channels.

Motility

Cell motility increases with the rate of malignancy but can also be stimulated by adding EGF to the cells' environment [13, 14, 103-107]. To date we do not understand by which mechanism the EGF enhances motility. The modulation of BK channels through EGF-mediated $[Ca^{2+}]_i$ oscillations could be the mechanism for EGF induced glioma motility as it might facilitate periodic cell swelling and shrinking. It is thus possible that BK channels are also involved in cell locomotion. On the other hand EGF might also halt glioma migration if it premanently activates BK channels and induces cell proliferation ('go *or* grow') like it was seen with acetylcholine [46]. What effect exactly EGF has on 1321N1 cells in unknown.

2 Aim of the study

The human astrocytoma cell line 1321N1, which is characterized by a stable expression of BK channels, was investigated. One aim was to characterize these channels electrophysiologically and study their pharmacology. A second aim of this study was to investigated whether BK channels are active at physiological membrane potentials in 1321N1 astrocytoma cells because this is a prerequisite for a functional role of these channels. Furthermore it was of interest to which extent BK channel gating would be facilitated by physiological $[Ca^{2+}]_i$. Moreover the functional role of BK channels is unknown to date. In order to investigate the putative role of BK channels for the tumor biology, we tested the effect of BK channel blockers on the proliferation, motility and transwell filter permeation (volume regulation) of human glioma cells. The study was also aimed to determine which types of growth stimulatory mediators excert their effect through BK channel modulation. Especially the effects of moderately elevated $[K^+]_e$ on one hand and chronic exposure to EGF on the other hand on proliferation and electrophysiological properties of an astrocytoma cell line were investigated.

3 Materials and Methods

3.1 Cell culture

1321N1 cells were purchased from the European Collection of Cell Cultures (Salisbury, U.K). Cells were cultured in 5 ml culture flasks (Greiner, Germany) with Modified Essential Medium (MEM). The Medium contained Earls Salts, L–glutamine, 10 % Fetal Calf Serum (FCS) and 1 % gramicidin. A change of the culture medium was performed every 48 hours. Cultures were kept at 37 C in a fully humidified atmosphere of 5 % CO_2 in air. For both electrophysiological and cell proliferation studies cells cells from in between passage 10 and 20 were used.

3.2 Proliferation assay

For proliferation experiments cells were seeded in 24 well tissue plates at a density of 20,000 per well. For 12 hours cells were allowed to become adherent in 10 % FCS. For the following days cells were incubated in medium containing either 5 or 20 mmol K^+ with or without BK channel blocker (100 nM Ibtx or 1 mM TEA). High K^+ medium was established by adding 10µl of a sterile 1 M KCl stock solution per 10ml medium. 10µl of a 10µM IBTX stock solution were or 10µl of a 100 mM TEA stock solution were added respectively. The modified medium was changed daily. After 6 days cells were trypsinized (0,2 % Trypsine) and resuspended in 1 ml MEM. Cells were counted under a light microscope with 10x magnification in a Fuchs – Rosenthal chamber. Per well five squares of the chamber were counted. In the experiments where blockers were applied all values were normalized to the control value in order to show the cell number in presence of a blocker as percentage of the controls. Statistical significance was assumed if the means were statistically different at the 0.01 level. Each experiment was performed three times.

3.3 Electrophysiological investigations

For all experiments cells were plated on untreated glass coverslips at a density of 40,000 per well and were allowed to become adherent in 10 % FCS for 12 hours. All measurements were performed between the days 2 – 6. The medium was changed every 48 hours.

Single-channel currents and whole-cell currents were measured with the patch clamp technique. Patch pipettes were pulled from borosilicate glass with a 2 step-puller (Narishige) and polished to a resistance of 3 – 5 MΩ.

All signals were low-pass filtered at 10 kHz and digitized with a sampling rate of 50 kHz. Pulsing and recording were performed with the ISO2 software (MFK, Niedernhausen, Germany). The cell capacitance was determined with ramp pulses using a routine implemented in the software.

3.3.1 Whole-cell recordings

The bath solution for whole-cell recordings contained (in mM): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4 and 10 glucose. IBTX (1µl/ml) and TEA (1µl/ml) were added from 10 µM and 100 mM stock solution to yield final concentrations of 100nM and 1mM, respectively. IBTX was obtained from Bachem (Germany) and TEA was obtained from Sigma (Germany).

Pipettes were filled with a solution composed of (in mM): 130 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.4. Therefore a free [Ca^{2+}] of 10 nM was calculated. Alternatively a pipette solution with 1 µM free Ca^{2+} was used which had the following composition (in mM): 130 KCl, 5 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes and 1,09 EGTA. To supress all K+ outward currents the pipette solution contained 130 mM CsCl instead of KCl **(Figure 4).**

3.3.2 Single-channel recordings

Determination of the unitary slope conductance was done in symmetrical KCl solution. The pipette solution was the same as for whole-cell experiments.

The bath solution contained (in mM): 130 KCl, 5 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose.

In the cell-attached configuration current traces of 100 ms duration were recorded at different pipette potentials. The mean current was calculated for each pipette potential from at least 20 current traces. **(Figure 4)**

3.3.3 Excised-patch recordings

The pipette solution for inside – out experiments contained (in mM): 130 KCl, 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES, pH 7.4. The bath solution contained 13KCl, 117 NaCl, 10Hepes and 1EGTA, pH 7.4. The bath solution with 100 nM free [Ca^{2+}] was established by adding 141µl of a 0.1M $CaCl_2$ stock solution per 100 ml. The bath solution with 1µM free [Ca^{2+}] was established by adding 944µl of an 0.1M $CaCl_2$ stock solution per 100 ml. In the bath solution with 1mM free [Ca^{2+}], EGTA was omitted and 0.1ml of an 1M $CaCl_2$ stock solution was added per 100 ml. The open probabilities were obtained by calculating the average current in 100 traces of 100 ms duration and relating this current to the maximum current at the high [Ca^{2+}] of 1mM **(Figure 4).**

3.3.4 Data analysis

Currents were analyzed with the ANA3 software (MFK Niedernhausen, Germany). Statistical calculations and curve fits were done with Origin (Version 6.1). Graphs were created with Corel (Version 9).
All data are presented as means +/- SEM unless noted otherwise.
The concentration of half maximal block (IC_{50}) of the whole-cell current by TEA was obtained by fitting the Hill equation

$$I/I_{max} = (1 - A) / (1 + ([TEA] / IC_{50})^{n} + A \qquad (1)$$

to the data points. I is the actual current, I_{max} the maximum current in the absence of TEA, A is a parameter for the unblockable current component, n is the Hill coefficient, IC_{50} is the [TEA] generating half maximal block.
Half maximal activation with 10 nM and 1µM pipette Ca^{2+} as well as half maximal activation of currents after chronic EGF exposure was calculated according to Tsen – Crank [74]. To begin with the I/V curves for each patch are plotted from the

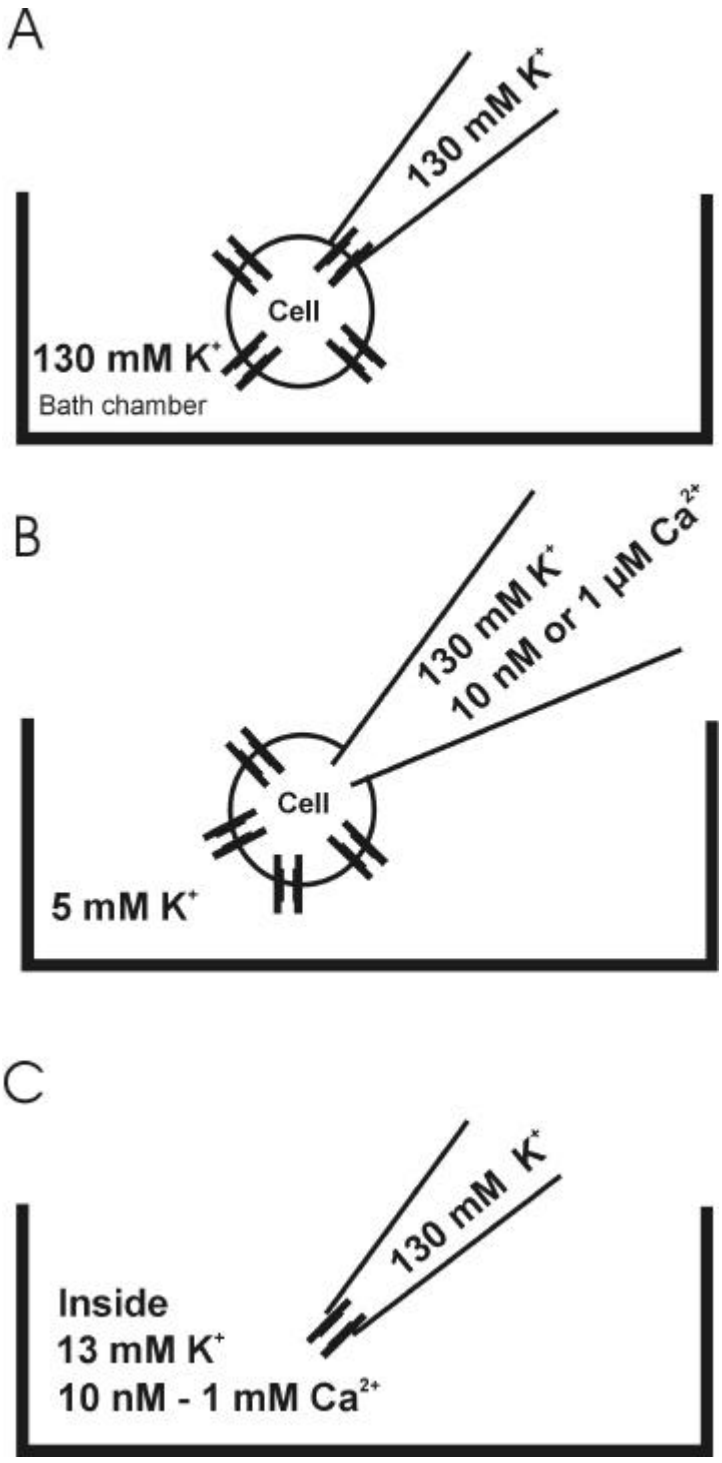

Figure 4 – Synopsis of patch – clamp techniques employed in this study.
A) Cell attatched recording under depolarizing conditions (chemical clamp) in symmetrical $[K^+]$. Under these conditions the actual membrane potential is equal to the clamped potential.
B) Whole cell recording. $[Ca^{2+}]_i$ is either 10nM or 1µM.
C) Excised patch recording. $[K^+]$ in the bath and in the pipette were chosen so that log $[K^+]_i / [K^+]_o = -1$. The pipette solution contained 130 mM KCl and the bath solution contained 13 mM KCl. The resulting equilibrium potential was calculated as +58 mV.

measured peak currents at each clamped voltage. Then the conductance G is calculated with the following formula :

$$G = I / (V_m – V_{rev}) \quad (2)$$

V_m is the clamped potential and V_{rev} is the calculated reversal potential under the imposed ionic gradients (- 84mV). These data are again plotted.
Following plotting they are fit with a Bolzman function (3) to estimate G_{max}.

$$y = (A1 – A2) / (1 + e^{(x+xo)/dx}) + A2 \quad (3)$$

A1 is the bottom asymptote and A2 is the top asymptote.
From these data the normalized conductances (G/G_{max}) are calculated. These values are plotted against the clamped potential and then again fitted using a Bolzman function. This last operation yields the voltage of half maximal BK channel activation ($V_{½}$). [74]

3.4 Migration studies

3.4.1 Boyden-Chamber migration assay

The bottom compartments of the dishes (Corning – Costar, Germany) were incubated with 600 µl of standard MEM with 10 % FCS with or without blocker. A cell suspension with a density of 40,000 cells/ml was filled into the upper compartment either with or without addition of a blocker. Cells were allowed to migrate for 12 hours. Then the membrane was washed with PBS, incubated in May–Grünwald stain for 5 minutes, washed again with PBS and finally incubated in Giemsa stain for 20 minutes. Filter membranes were then left to dry for 48 hours after which they were cut out with a scalpell and embedded in neutral balsam (xylol based) on a glass side. In each experiment 5 control wells were compared to 5 wells with blocker. Each experiment was repeated three times. For each well 5 fields were counted under a

microscope with 10 x magnification using a standard calculator to facilitate counting. Means were normalized with respect to the controls.

3.4.2 Time-lapse videomicroscopy

Cells were seeded into 8 cm Petri dishes in a density of 5,000 cells/ml and allowed to adhere for 12 hours. Then they were placed under the video–microscope and monitored for 12 hours. Per dish five fields could be monitored during the period and the distance the cells had travelled were later measured offline with standard software. For each channel modulator 20 cells were studied. 20 control cells incubated in standard MEM with 10 % FCS were also studied.

3.5 Statistical analysis

To test for significant differences we used a non-paired t test ($p < 0.01$). Statistical analysis was performed with the software Origin v.6.1 (Microcal).
For proliferation experiments, Transwell assays and timelapse studies statistically significant differences were defined if a non–paired *t*–test yielded significantly different means at a $p < 0.01$.

4 Results

4.1 The human astrocytoma 1321N1 cell line display different morphological cell types in culture

After seeding the cells in the presence of 10 % FKS for 24 hours cells became adherent to the glass coverslips. There were two types of cells, i.e., (i) cells of a flat, fibroblast like shape and (ii) round cells without processes. After 48 hours in culture the majority of cells were flat and only very few round cells were interspersed. The same picture was observed whether cells were grown with serum or under serumfree conditions. Even under serumfree conditions cells remained viable for at least 6 days. **(Figure 5)**

4.2 Human astrocytoma 1321N1 cells show BK currents, which are active under physiological conditions

4.2.1 Whole-cell recordings

All control measurements were performed on cells grown for 2 to 6 days under serum free conditions (n=30).

The resting membrane potential was measured after switching to the current clamp mode as being - 23 +/- 2 mV (n=30). Series resistance and cell capacitance were recorded in the voltage clamp mode. Values obtained were 919 +/- 122 MΩ for the membrane resistance (n=30) and 27 +/- 1 pF (n=30) for the cell capacitance. With 10 nM pipette [Ca^{2+}] and voltage steps from – 60 mV to + 180 mV at a holding potential of – 60 mV control cells (n=30) displayed a uniform activation course of voltage-dependent outward currents. These currents activated rapidly within less than 10 ms and did not deactivate in the 100 ms pulse. Strong depolarizations were necessary to elicit them as they activated at values > + 60 mV only (n=30) **(Figure 6)**.

The currents were established to be K^+ currents by using CsCl as pipette solution that completely abolished the K^+ currents **(Figure 6)**.

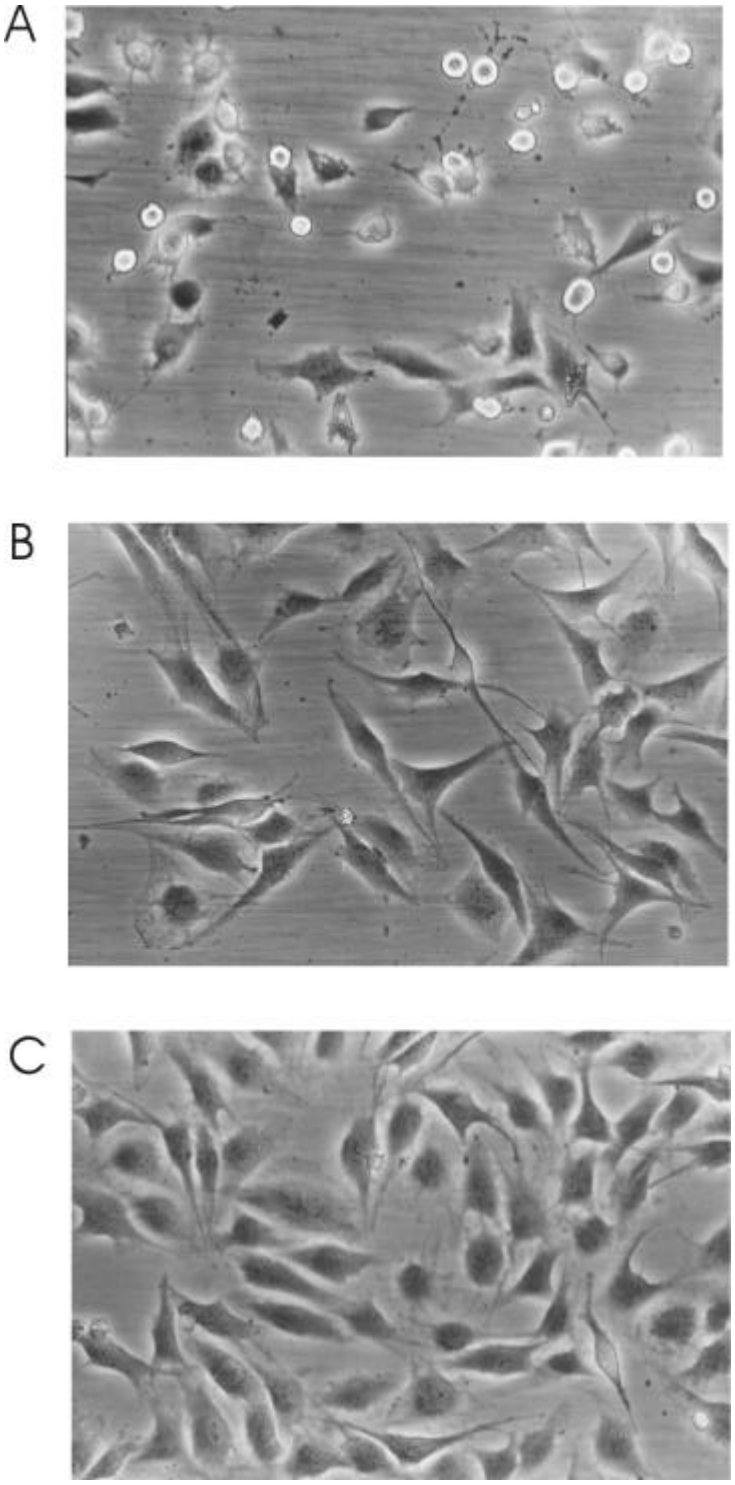

Figure 5 – Morphology of 1321N1 cells during 6 days in culture.
After culturing the cells for 24 hours in the presence of 10 % FBS most of the cells adhered to the glass coverslips. Early serum depriviation prevents adhesion.
A) During the initial 48 hours in culture many round cells are found.
B) After this period most of the cells change their shape to a flat shape with long processes.
C) After 6 days in culture in the presence of 10% FBS the cell density increases markedly. Under serum free conditions the same morphological picture is observed but the cell density does not increase as much as in the presence of 10% FBS.

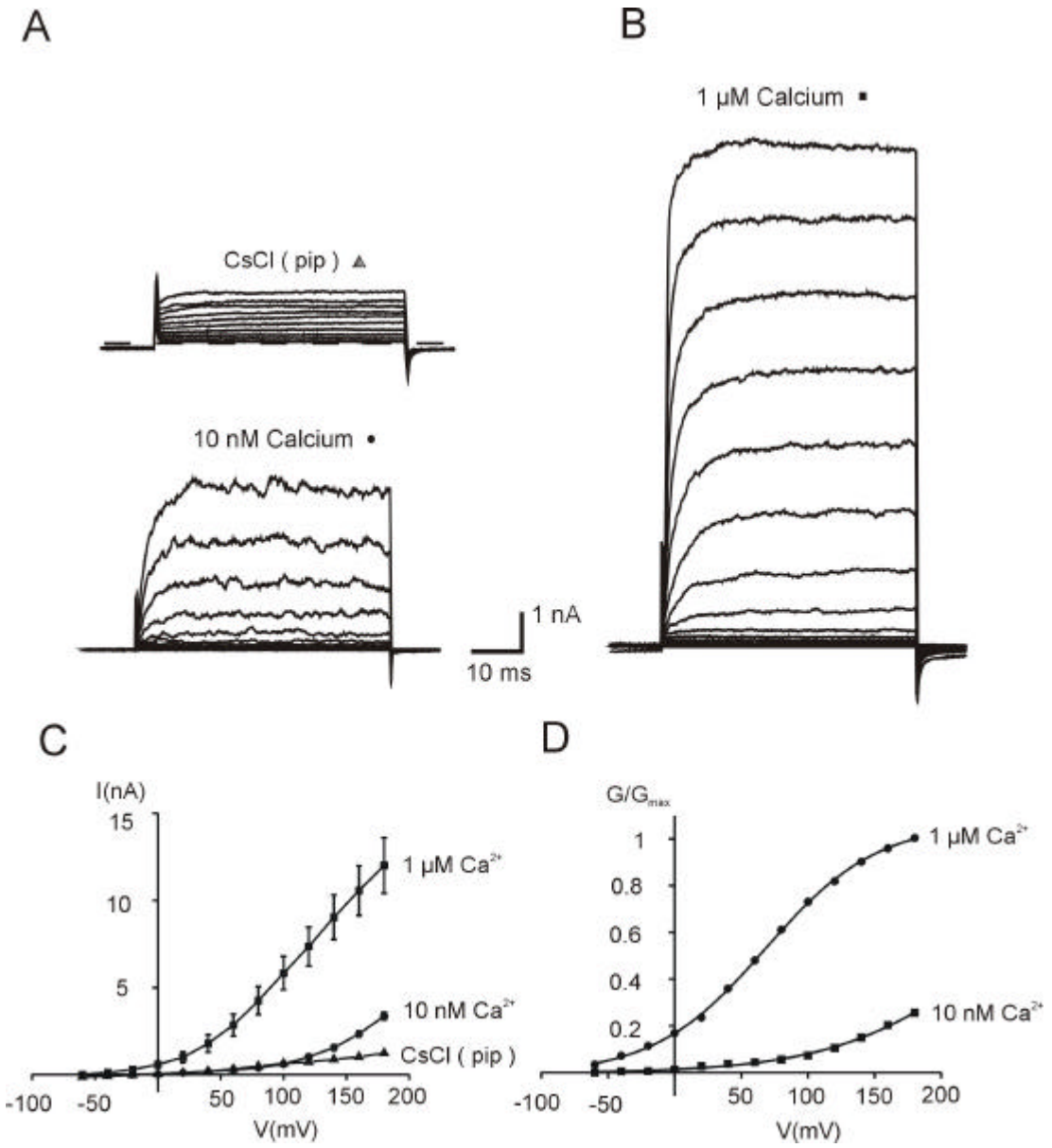

Figure 6 – Basic properties of voltage dependent currents in 1321N1 cells.
A) Whole cell recordings from a single 1321N1 cell with a 10 nM KCl or a CsCl based pipette solution. K^+- outward currents were abolished by CsCl.
B) With 1 µM $[Ca^{2+}]_i$ the current amplitude was markedly increased and the current noise was reduced.
C) I -V plot of the steady state currents illustrated in A and B. With 10 nM $[Ca^{2+}]_i$ the voltage dependent currents activated only at potentials > 50 mV (n = 28). With 1 µM $[Ca^{2+}]_i$ the I-V curve was shifted to the left (n = 6).
D) Normalized conductances (G/Gmax) are plotted against the membrane potential for 10 nM (n = 30) and 1 µM (n = 6) $[Ca^{2+}]_i$. The curves were constructed as described in Materials and Methods. The datapoints were fit with a Bolzman function (3). Halfmaximal activation ($V_{½}$) occurs at 243 ± 72 mV with 10 nM $[Ca^{2+}]_i$ (n = 28) and 68 ± 1 mV at 1 µM $[Ca^{2+}]i$ (n = 6).
All recordings were performed with voltage steps of 50 ms duration from – 60 mV to + 180 mV at a holding potential of – 60 mV.

$[Ca^{2+}]_i$ in glioma cells was reported to lie between 10 nM and 1 µM [46]. We therefore investigated I/V relationships with the two different $[Ca^{2+}]_i$ (10 nM and 1 µM) in the pipette solution. All recordings were started later than 3 minutes after formation of the whole-cell patch to allow Ca^{2+} to fully equilibriate between pipette and cell interior.

With 1 µM Ca^{2+} the current noise was markedly decreased. Since both mean open time and open probability depend on Ca^{2+} this may reflect a stabilization of the open state with increasing intracellular Ca^{2+} [87].

We calculated points of half maximal activation applying the method described by Tsen–Crank et al. [74] (see Materials and Methods). With 10 nM intracellular Ca^{2+}, the voltage at which channels were 50% open ($V_{½}$) was + 241 ± 72 mV (n = 28) with 1 µM intracellular Ca^{2+} half maximal activation occurred at + 68 ± 1 mV (n = 6). The voltage required to activate the BK channels was 42 ± 1 mV per e-fold increase of the current at 1 µM internal Ca^{2+} and 59 ± 11 mV e-fold increase of the current at 10 nM internal Ca^{2+} (slope of the Boltzmann fit).

Next we tested the ability of BK channel blockers to inhibit the whole-cell currents of 1321N1 cells. We used the specific BK channel blocker IBTX, a scorpion toxin first described by Galvez et al. [108] and TEA in a concentration of 1 mmol/l where it is a specific blocker of BK channels [109]. Whole-cell currents were elicited from a holding potential of –60 mV by pulses to voltages between –180 and +180 mV in 20 mV steps before and after bath application of IBTX **(Figure 7A).** The IV-relationship constructed from the steady-state current amplitudes during the test pulses shows an inhibiton of outward currents in the presence of 100 nM IBTX, especially at potentials >+80 mV. IBTX inhibited the outward currents by 56 +/- 4 % at 140 mV; 61 +/- 4 % at 160 mV and 65 +/- 4 % at 180 mV (n = 10). The IBTX block was completely reversible after several minutes washout time.

Figure 7B shows the inhibitory action of various concentrations of tetraethylammonium (TEA) at the potential of +180 mV. 1 mM TEA blocked the currents by 69 +/- 12 % at 140 mV, 70 +/- 9 % at 160 mV and 71 +/- 6 % at 180 mV (n = 3). In the presence of 1 mM TEA the current noise was greatly reduced together with the current amplitude. This is consistent with less opening events of large conductance channels. The effects of TEA were completely reversible after complete washout of the drug. An increase of TEA to 10 mM did not further reduce the current amplitude.

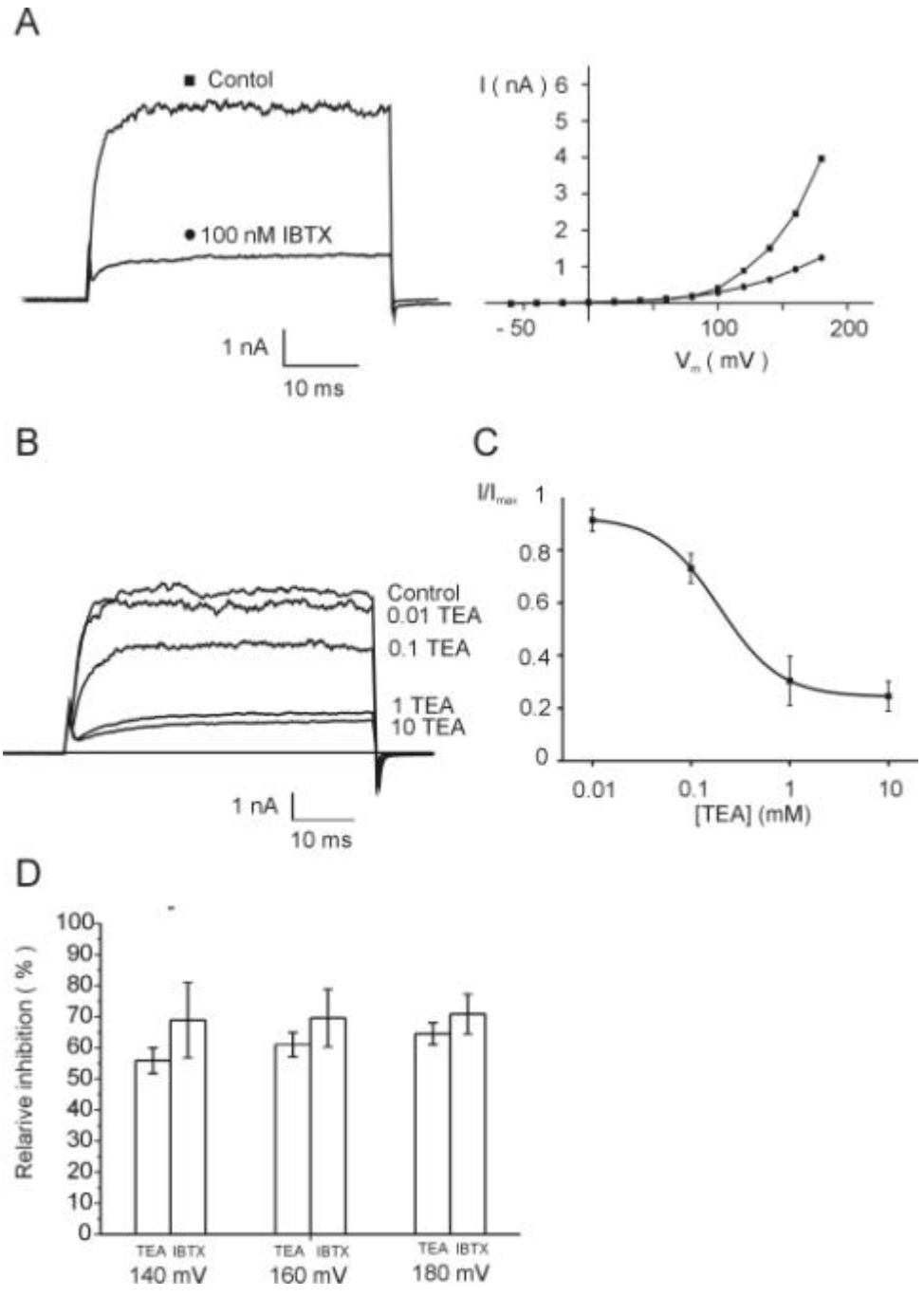

Figure 7 – Pharmacology of BK currents in 1321N1 cells.

A) Whole cell currents and I-V relation from a single 1321N1 cell were inhibited > 70 % by the specific BK channel blocker IBTX in a concentration of 100nM.

B) Whole cell currents from a single 1321N1 cell were blocked with 0.01, 0.1, 1 and 10mM TEA respectively. TEA 1 mM blocked the currents by > 70 %.

C) Dose response curve for BK current inhibition by TEA in n=3 cells at +160mV. Data points are means +/- SEM of the steady state current amplitude. Fitting the datapoints with the Hill equation (1) yielded an IC_{50} of 0.19mM TEA.

D) BK current block by neither IBTX nor TEA 1 mM is voltage dependent. There were also no statistically significant differences in the amount of block between the two drugs (IBTX : n = 10, TEA : n = 3).

All whole-cell currents were elicited from a holding potential of -60 mV by pulses of 50 ms duration to voltages between –60mV and +180 mV in 20 mV steps.

Figure 7C illustrates the dose-response relation of TEA-induced reduction of outward currents in 1321N1 cells (n = 3). The concentration of half maximal block (IC_{50}) was obtained by fitting the Hill equation (1) to the data points. The IC_{50} was determined to be 0.19 mM. A similar voltage dependence of outward currents was observed in cells from other gliomas [4] and meningiomas [86] also at lower intracellular [Ca^{2+}] of 10 or 20 nM.There was no significant difference in between 1mM TEA and 100 nM IBTX potency at any voltage.

In summary, the electrophysiological recordings and pharmacological experiments identify outward currents to be mediated by BK channels.

4.2.2 Single-channel recordings

Single-channel currents in **cell-attached (n = 10) and inside-out patches (n = 3)** from 1321N1 cells were investigated. With cell-attatched patch recordings the unitary slope conductance and the open probability at a certain clamped potential can be determined. Furthermore with this technique the cell remains intact and the channel activity at the (unknown) physiological [Ca^{2+}]$_i$ can be recorded. Inside out patch recordings alow to study BK channel activity at the physiological membrane potential while at the same time [Ca^{2+}]$_i$ (the [Ca^{2+}] in the bath solution) can be varied as it is desired.

Cell-attached recordings

For cell attatched recordings cells (n=10) were bathed in a solution containing a high [K^+] (130 mM) which depolarizes the resting membrane potential toward values of about 0 mV (chemical clamp) [3, 110]. In other words: with such a depolarizing K^+ solution little potential difference remains across the membrane because the chemical clamp eliminates any membrane potential generated by the cells K+ channels. The actual membrane potential (V_m) then is equal to the clamped potential added to the reversal potential for K+ (- 84 mV).

Figure 8 shows the activity of BK channels in a cell-attached patch at depolarizing pulses. These channels were found in all patches (n = 10). In cell-attached recordings currents were activated only at potentials positive to + 50 mV very similar to what can be observed in whole-cell experiments with 10 nM internal Ca^{2+}. The unitary current was calculated by a Gaussian fit of amplitude histograms by means of

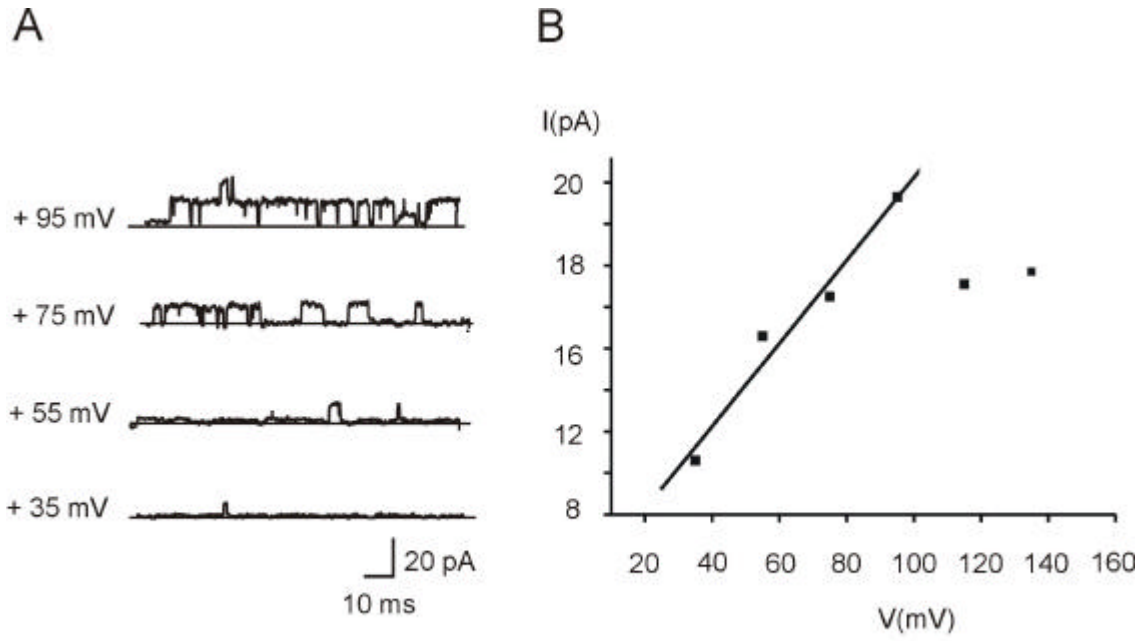

Figure 8 - Properties of single BK channels in 1321N1 human astrocytoma cells.
A) Example of a cell-attached patch in a single cell at different depolarizing pulses from a holding potential of 0 mV at depolarizing conditions with a 140 mM KCl bath solution. The solid line marks the closed level.
B) Single channel current amplitudes recorded from 10 cell-attached patches were averaged and plotted as function of voltage. The IV-relation is linear for potentials between +35 and +95 mV resulting in a slope conductance of 209 +/- 28 pS. The reversal potential was experimentally determined to be + 4 +/- 2 mV.

the ANA3 software. For each voltage a current can be plotted. The slope conductance was calculated by fitting the datapoints in the linear part of the I/V relation.

We calculated a unitary slope conductance of 209 +/- 28 pS from single channel recordings (n = 10). The reversal potential was experimentally determined to be + 4 +/- 2 mV (n = 10). The theoretical reversal potential for symmetrical solutions would be 0 mV. The current–voltage relation is not linear at potentials > 120 mV as would be expected from Ohms law. At voltages > 140 mV the conductance decreases and finally even becomes negative. This is possibly an effect of a voltage dependent channel block by internal ions [110] **(Figure 8)**.

Excised-patch recordings

Excised-patch recordings were performed to demonstrate that BK channels are also active at the physiological membrane potential of – 20 mV and at physiological intracellular Ca^{2+} concentrations. The channel activity was determined with different Ca^{2+} concentrations in the bath. The resting membrane potential were determined previously in control cells (n = 30). The value was –23 ± 2 mV. In the excised patches the channel activity was recorded during pulses to –20 mV of 100 ms duration elicited from a holding potential of 0 mV. The pipette solution contained 130 mM KCl and the bath solution contained 13 mM KCl. The theoretical equilibrium potential for these ion concentrations was calculated to be +58 mV. Under these conditions it was possible to determine the channel activity at the normal resting potential of –23 mV while variing the intracellular free Ca^{2+} within the physiological range. Four different $[Ca^{2+}]_i$ were tested: a Ca^{2+} free solution, as well as solutions 100 nM, 1 µM and 1 mM free Ca^{2+}. When the Ca^{2+} free solution was added the channels closed completely after complete washout of Ca^{2+}. The activity rapidly recovered after one of the solutions containing Ca^{2+} was added. For 0 Ca^{2+} the open probability was 0.0013 +/- 0.0008 %, for 100 nM 0.0060 +/- 0.0032, for 1 µM 17.99 +/- 4.89 % and for 1 mM 81,20 +/- 2.20 %. **Figure 9** shows that within the range of physiological intracellular Ca^{2+} concentrations (100 nM – 1 µM) there is a significant BK channel activity in resting 1321N1 cells **(Figure 9)**.

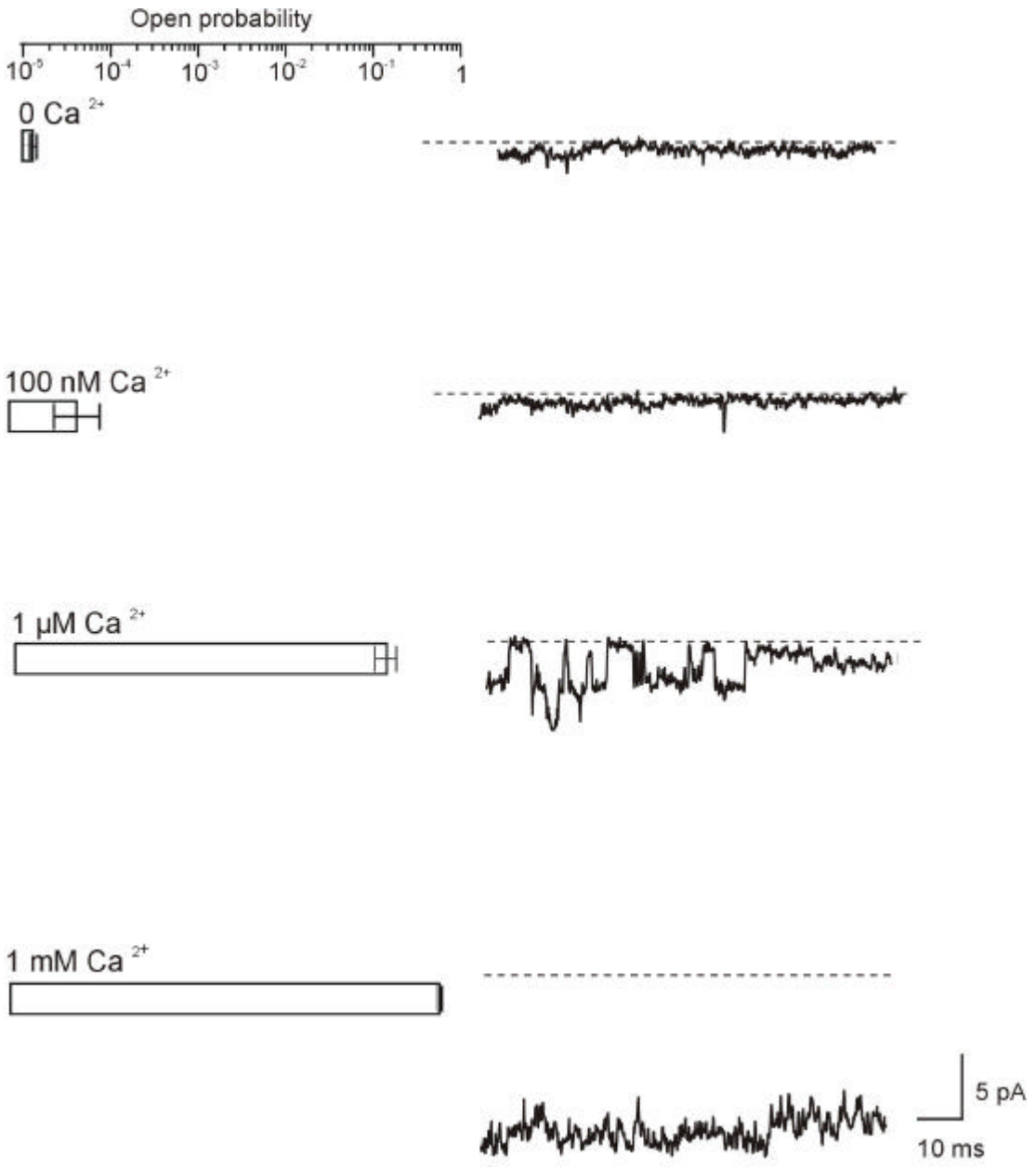

Figure 9 – Excised patch recordings.
The channel activity in three inside-out patches was recorded at -20 mV using an intracellular bath solution containg 13 mM K^+ and different [Ca^{2+}]. Within the range of physiological [Ca^{2+}]$_i$ (100 nM – 1 µM) there is a significant BK channel activity in resting 1321N1 cells.

4.3 Chronic exposure to EGF depolarizes 1321N1 cells and increases K^+ outward currents.

To investigate whether EGF like in other cells modulates BK mediated currents in 1321N1 cells we cultured the cells for 4 – 6 days in serumfree medium with or without 10 ng/ml EGF respectively. We observed that the cells exposed to EGF (n = 24) were depolarized in comparison to the controls ($[Ca^{2+}]_i$ = 10 nM; n = 28). The mean value of the membrane potential was - 23.2 ± 2.1 mV for the control cells vs. - 16.2 ± 1.7 mV for the EGF treated cells ($p < 0.01$). Whole-cell currents were elicited from a holding potential of -60 mV by pulses to voltages between -180 and +180 mV in 20 mV steps. The IV-relationship constructed from the steady-state current amplitudes during the test pulses shows an augmentation of outward currents after chronic exposure to EGF, especially at potentials >+ 80 mV compared to the controls. At the potential of +180 mV normalized currents were 105 ± 12 pA/pF in the controls and 264 ± 45 pA/pF in EGF treated cells which is an increase by 151 % in EGF treated cells.

We calculated points of half maximal activation of BK channels for untreated cells at a $[Ca^{2+}]_i$ = 10 nM and for cells, which were treated with EGF also at a $[Ca^{2+}]_i$ = 10 nM applying the method described by Tsen–Crank et al. [74] (see Materials and Methods). After chronic EGF treatment the voltage at which channels were 50% open (V ½) was + 130 +/- 1 mV (n = 24) whereas in the controls half maximal activation occurred at + 241 ± 72 mV (p = 0.001). The voltage required to activate the BK channels was 26 ± 1 mV at 1 µM internal Ca^{2+} and 59 ± 11 mV in the controls (slope of the Boltzmann fit) **(Figure 10).**

4.4 Under particular conditions specific BK channel blockers inhibit proliferation of 1321N1 astrocytoma cells

In a first set of experiments we investigated whether different culture conditions modified the growth rate of 1321N1 astrocytoma cells. We found that serum increased proliferation by 41 +/- 2 % as compared to cells grown in serumfree media (p = 0.01). Moreover, when the media contained 20 mM instead of 5 mM extracellular K^+, cell proliferation was increased by 21 ± 3 % (p = 0.01) **(Figure 11).**

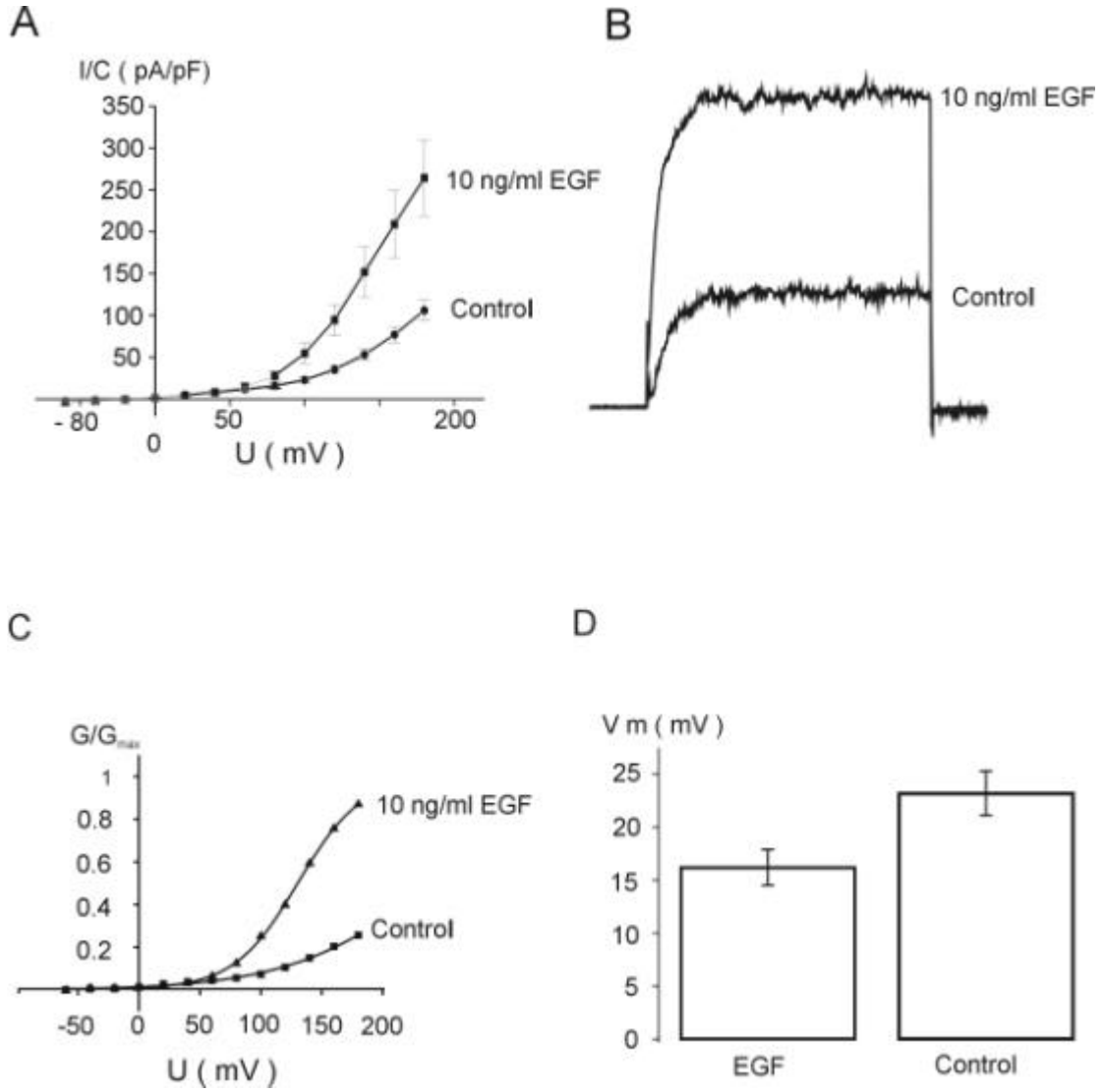

Figure 10 – The effect of prolonged (6d) EGF exposure on K^+channel activity

Whole-cell currents were elicited from a holding potential of -60 mV by pulses of 50 ms duration to voltages between -60 mV and +180 mV in 20 mV steps.

A) IV relations of cells grown in the presence of 10 ng/ml EGF (■) (n = 24) and in its absence (●) (n = 28). Cells grown for 6 days under serumfree conditions show a marked increase of voltage-dependent K^+- outward currents when 10 ng/ml EGF was added during the cultivation period. Peak currents at + 180 mV were 105 ± 12 pA/pF for the controls and 264 ± 45 pA/pF in EGF treated cells (151 % increase).

B) Whole cell recording of a control cell cultured for 6 days under serum free conditions compared to a whole cell recording of a cell cultured for 6 days in the presence of 10 ng/ml EGF

C) Conductance voltage relation of cells grown in the presence of 10 ng/ml EGF (▲) and in its absence (●). Half maximal activation of these currents ocurred at much more positive potentials after cultivation in the presence of EGF (+ 130 +/- 1 mV) than in its absence (+ 241 ± 72 mV).

D) Chronic exposure to EGF causes a marked membrane depolarization of 1321N1 cells. The mean value of the membrane potential was - 23.2 ± 2.1 mV for the control cells vs. - 16.2 ± 1.7 mV for the EGF treated cells.

Moderate elevation of $[K^+]_e$ is thus a potent stimulus for the proliferation of astrocytoma cells. This K^+ dependent increase of proliferation could be completely abolished by the BK channel blockers 1 mM TEA and 100 nM IBTX. Data from three experiments were pooled and yielded a total inhibtion of proliferation by IBTX of 20 ± 3 %. All three experiments were significantly different in themselves when compared to controls (p = 0.01). Comparable results could be demonstrated with 1 mM TEA; the total inhibition of proliferation with 1 mM TEA for the three experiments was 30 ± 4 % (p = 0.01). Very strikingly, BK channel blockers were, however, ineffective when the medium contained only physiological $[K^+]_e$ (5 mM) instead of 20 mM $[K^+]_e$ (n = 3) (p = 0.01). The proliferation was increased only at moderately elevated $[K^+]_e$ of about 10 – 30 mM but was significantly inhibited at concentrations above 50 mM. In single experiments we tested the influence of different $[K^+]_e$ of 10, 40 and 100 mM on the proliferation of 1321N1 cells. Whereas 10 mM $[K^+]_e$ increased the proliferation by about 4%; 40 mM and 100 mM $[K^+]_e$ decreased the proliferation by about 19% and 75%, respectively.

4.5 Specific block of BK channels does not influence migration of 1321N1 astrocytoma cells

4.5.1 Boyden chamber migration assay

To investigate whether BK channels have a role in the migratory potential of human glioma cells we first used the Boyden chamber migration assay (Transwell assay).
Cells were plated on the upper surface of Transwell membranes with 8 µm pores. Migration was allowed to take place for 12 hours away from the high cell density in the upper compartement towards the lower compartment, which contained no cells. Channel blockers (IBTX 100 nM, 1mM TEA, 5 mM TEA) were dissolved in sterile destilled water and added to the upper as well as to the lower compartment. Sterile destilled water was added to the controls. All migration media contained 5 % FCS.
For each well 5 HPF (10 x) were counted in the lower compartment **(Figure 12A).** Neither 100 nM IBTX nor 1 mM TEA had any effect on cell invasion in the Transwell migration assay **(Figure 12)**.

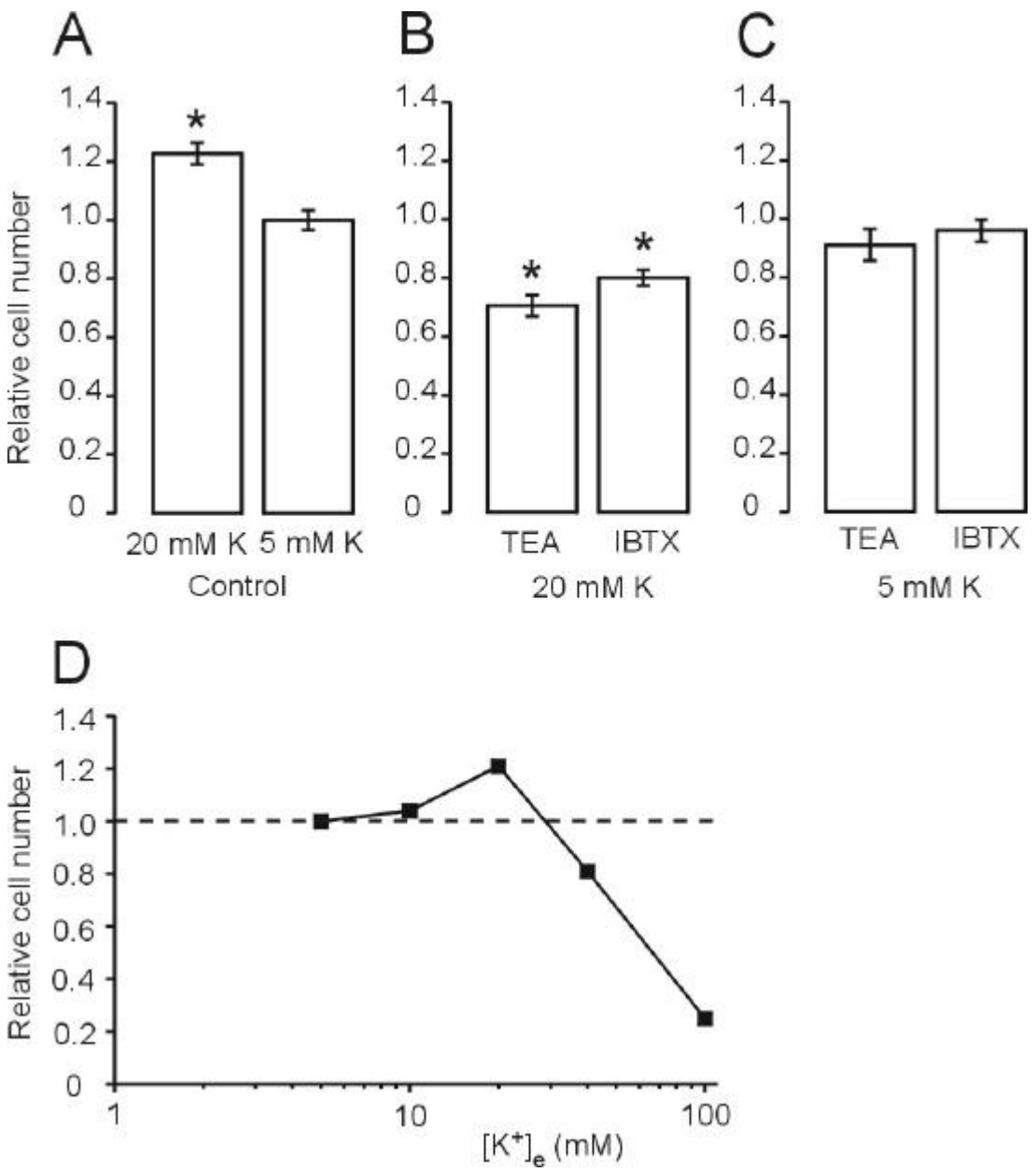

Figure 11 - IBTX and TEA inhibit proliferation of 1321N1 cells at elevated (20 mM) $[K^+]_e$.

A) An increased $[K^+]_e$ in the culture medium stimulates the proliferation of 1321N1 cells. The relative cell number at $[K^+]_e$ = 20 mM is 123 ± 4% in comparison to the cell number at $[K^+]_e$ = 5 mM. (∗ signifies $p < 0.01$; The error bars are ± SEM).

B) IBTX (100 nM) and TEA (1 mM) reduce the proliferation at $[K^+]_e$ = 20 mM by 21 ± 3% and 30 ± 4%, respectively (∗ signifies $p < 0.01$ The error bars are ± SEM).

C) IBTX and TEA have no effect on the proliferation at $[K^+]_e$ = 5 mM (the error bars are ± SEM).

D) The proliferation is increased only at moderately elevated $[K^+]_e$ of about 10 – 30 mM. The relative cell number at $[K^+]_e$ = 5 mM and $[K^+]_e$ = 20 mM were taken from (A). Data at $[K^+]_e$ of 10, 40 and 100 mM were taken from single experiments.

Cell numbers counted were 274 +/- 16 cells per HPF for the control group and 279 +/ - 19 cells per HPF for the group exposed to 100 nM IBTX (p = 0.01). For the experiment with 1 mM TEA values were 431 +/- 15 cells per HPF for the control versus 440 +/- 20 cells per HPF for the TEA 1 mM group (p = 0.01) TEA was however effective in a concentration of 5 mM. Cell numbers were 597 +/- 17 cells per HPF for the control group versus 283 +/- 17 cells per HPF in the group exposed to 5 mM TEA (p < 0.01) **(Figure 12C)**. Mean inhibition from 6 wells was 52 +/- 6 %.

4.5.2 Time–lapse videomicroscopy

To further dissect tumor cell migration and the role of BK channels we studied the effects of IBTX (100 nM) and TEA (1, 5 and 20 mM) on migration velocity with a time–lapse videomicroscope.

After allowing 1321N1 cells to become adherent for 30 h in a 8 cm Petri dish we applied fresh medium containing 5 % FCS and added 100 nM IBTX, 1 mM TEA, 5 mM TEA or 20 mM TEA in destilled water or destilled water alone to the controls.

Per dish 5 cells could be studied for 6 hours. For each blocker 20 cells were recorded and compared to a single control group of 20 cells. The mean distance travelled during the 6 hours in the control group was 261.3 +/- 17.5 µm. There was no significant difference to the IBTX group (238.5 +/- 25.0 µm; p < 0.01) **(Figure 13A)**. In the same experimental setting we also tested the effect of 1 mM TEA in the presence of which the cells travelled a mean distance of 213.3 +/-17.01µm. There was again no difference to the control group (p < 0.01). Migrational velocity, however, was slowed significantly when TEA doses > 5 mM were applied. With TEA at 5 mM mean distance travelled was 185.07 +/- 13.28 µm. This distance was significantly shorter than the distance travelled by the controls (p < 0.05). It meant a 29 +/- 5 % decrease in migrational velocity. With TEA at 20 mM the mean distance travelled was 168.97 +/- 16.5 µm. Compared to the controls this is a 35 +/- 6 % decrease in migrational velocity (p < 0.01) **(Figure 13A)**.

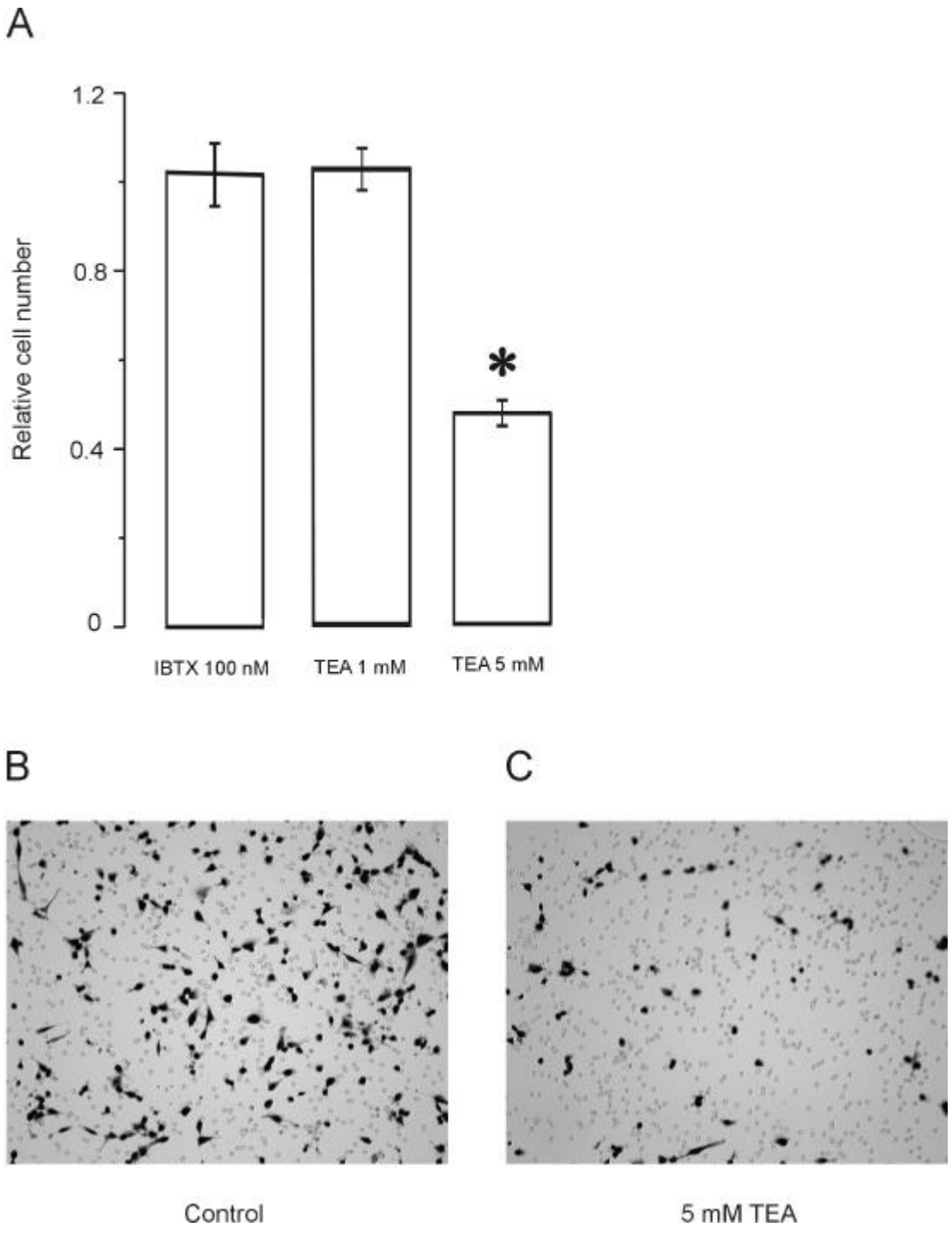

Figure 12 – Transwell migration assay.
A) Relative number of cells that have migrated through 8 µm pore size filters in the presence of a blocker relative to a control in the absence of the blocker. The y – axis represents the cell number on the lower filter surface normalized to the control. The data were averaged from three experiments. Only in the presence of [TEA] = 5 mM transwell migration was significantly inhibited whereas specific BK channel blockers were ineffective (∗ signifies $p < 0.01$; The error bars are SEM).
B,C) Representative microscopic fields of 1321N1 cells on the lower side of the membrane that have migrated through 8 µm pore size filters in the presence of 5 mM [TEA] (C) and absence of the blocker (B).

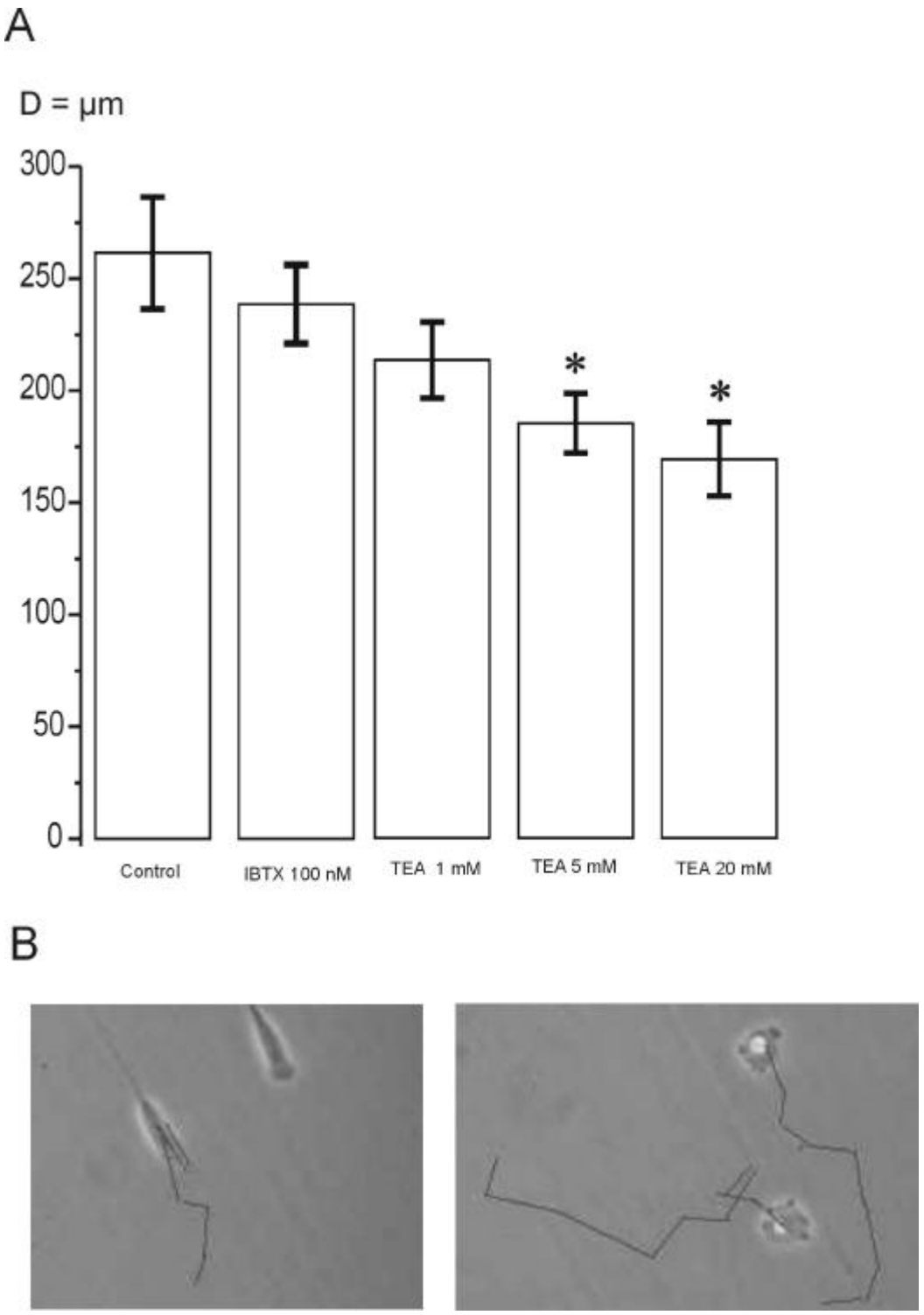

Figure 13 – Timelapse videomicroscopy.
A) The rate rate of cell migration in the plane is not diminished by specifically blocking BK channels with 100 nM IBTX (n = 20) or 1 mM TEA (n= 20). The velocity of 1321N1 cell migration is only diminished significantly with [TEA] = 5 mM (n = 20). [TEA] = 20 mM (n = 20) did not further slow migration. The y – axis is the distance (µm) the cells travelled in the 6h study period. The error bars are SEM (∗ signifies $p < 0.01$).
B) Two representative microscopic fields of 1321 cell movement tracked for 6h. The lines show the distance travelled by the cells during the 6h recording.

5 Discussion

5.1 BK channels in normal and neoplastic cells

BK channels are ubiquitous in excitable and nonexcitable cells. This underlines their fundamental role in coupling electrical and chemical signalling [88, 111]. In one study mRNA encoding for BK channels was found in eight examined representative regions of the human brain (amygdala, caudate nucleus, cerebellum, corpus callosum, hippocampus, substantia nigra, subthalamic nucleus) indicating that the channels are expressed in the entire brain [74]. Glial cells [112] as well as neurons [113-119] were observed to express BK channels. With radiolabelled probes which recognize all variants of the *hslo* gene – the gene that encodes for all human K_{Ca} channels – its expression was also detected in many nonneural tissues like the aorta, the spleen and the kidney [74]. Embryonic cells seem to also rely strongly on the activity of BK channels [120]. Moreover vascular smooth muscle cells, vascular endothelial cells [42, 91], pituitary cells [35] and adrenal chromaffine cells [121] are outfitted with BK channels. Human osteoblasts were also shown to have functional BK channels [122]. On the other hand little or no expression was evident in the liver, the lungs and the heart [74].

Besides in normal tissue the presence of such channels has been demonstrated in neoplastic cells. An example for a non-neuronal tumor whith BK channels is the rat insulinoma [121]. BK channels are also present in benign and malignant tumors of the CNS. They have been well characterized in meningeoma cells [86] and were also observed in cultured N1E-115 mouse neuroblastoma cells [87]. BK channels are an ubiquitous finding in malignant glioma cells. Several studies have described them in various glioma cell lines [3, 4, 123]. Here we showed that 1321N1 human astrocytoma cells are outfitted with BK channels.

While, as described above, it has been established that BK channels are common to almost all human tissue their functional role is only very vaguely characterized. Because these channels in some experimental settings were shown to require strong depolarizations for gating the question arises whether in nonexcitable cells they are at all functional. Furthermore it is unresolved whether these channels have a common cellular function irrespective of the cell type in which they occur, or whether they have specific functions in different cell types. Generally the question is whether

such an ubiquitous channel can have one single specific function in a certain subset of cells like neoplastic cells for instance. Even if these channels are ubiquitous they might be not functional and redundant in some cell types but essential for proper function or even survival in others. Therefore BK channels might well play an essential functional role in neopastic cells while being an item of peripheral importance in nonneoplastic cells.

5.2 Properties of BK channels in 1321N1 human astrocytoma cells

All K_{Ca} channels (SK, IK and BK channels) are encoded by a single gene on the long arm of the human chromosome 10 on band 10q22.3. This gene is known as the *slo* gene. The name is derived from a mutant form of Drosophila named Slowpoke where the gene was first identified. The mammalian variant is named *mslo* the human variant *hslo*. Several diverse mammalian BK channel types can be derived from this single gene by alternative splicing. The *hslo* mRNA family that encodes for BK channels in the brain is called hbr (human brain) and there are 9 isoforms (hbr1 – hbr9). Several isoforms were expressed in Xenopus oocytes and a significant variability in their electrophysiological properties was observed. For instance under identical conditions of Ca^{2+} and voltage the hbr5 ion channel had a much higher open probability than the hbr3 ion channel and it was 3 – 5 times more sensitive to Ca^{2+}. Thus a cell can effectively modify chemical and electrical signalling by selectively expressing various subforms of BK channels. Fine-tuning of a cells' chemical and electrical signalling cascades might be achieved when the various subtypes of these channels are coexpressed in a single cell [74]. This phenotypic pasticity of the channels explains differences in the properties of BK channels in 1321N1 cells and the properties of BK channels examined in other studies.

5.2.1 Ca^{2+}- and voltage-dependence of BK channels in 1321N1 human astrocytoma cells

The channels we characterised in 1321N1 cells displayed typical features of BK channels. During whole cell recordings at a holding potential of -60 mV when stepped from -80 to +180 mV 1321N1 cells showed a rapidly activating large outward

current that did not inactivate during the 100 ms pulse. The currents can be identified as K^+ outward currents since they were not diminished when chloride in the bath solution was replaced by glutamate and since they were completely suppressed with CsCl in the pipette solution.

Another typical feature of BK channels is their Ca^{2+} dependent gating. We performed recordings with 10 nM and 1 µM Ca^{2+} in the pipette because physiological $[Ca^{2+}]_i$ in malignant glioma cells have been previously reported to lie in this range [46]. Raising $[Ca^{2+}]_i$ from 10 nM to 1µM shifts the channels IV curve markedly to the left **(Figure 6C)**. BK channels therefore combine the properties of voltage- and ligand-gated ion channels. This raises the question whether both Ca^{2+} and depolarization are required to open the channel or whether only one of these suffices. Stefani et al. demonstrated that depolarization was able to open BK channels in a Ca^{2+} independent manner [83]. Therefore BK channels must have an intrinsic voltage sensor and it was speculated that voltage is the main activator of BK channels. The intrinsic voltage sensor is structurally similar to the voltage sensor in other voltage gated ion channels [124]. When micromolar Ca^{2+} is present BK channels switch from the Ca^{2+} independent mode to a Ca^{2+} modulated mode. In the latter mode much smaller gating currents were necessary to open the channels [83]. The channels Ca^{2+} sensitivity is determined by a separable C-terminal domain that is appended to the channels core and is unique to BK channels [125]. This separable domain is also referred to as the channels β – subunit [80, 88]. The position of the G-V relation along the voltage axis, which reflects the channels Ca^{2+} sensitivity is the only property, which is influenced by this subunit [88]. It was experimentally revealed that many different types of BK channels with very distinct properties can be assembled by combining different β -subunits with an α - subunit [125]. The channels other subunit is the α - subunit and is identical to the pore-forming subunit of other K+ channels [126]. Four of these subunits form the channel pore [124]. It does not necessarily need a β – subunit for a BK channel to function *in vitro* although the introduction of a β – subunit dramatically increases voltage and Ca^{2+} sensitivity. While it is clear that the regulation of the co-assembly will account for the functional diversity among members of the BK channel family the question remains whether *in vivo* the introduction of an β – subunit is optional [80].

A BK channel, which is insensitive to Ca^{2+}, was observed in mouse early embryos [120]. Moreover BK channels in aortal endothelial cells lack a β – subunit [127]. On the other hand vascular smooth muscle cells are composed of both α - and β - subunits [128, 129]. These data clearly indicate that co-assembly of an α and β - subunit is not obligatory for BK channel function *in vivo*. By regulating the β - subunit expression a cell can specify the physiological properties of its BK channels just as much as it can do by expressing various splice variants of the α - subunit. BK channels in 1321N1 cells are very sensitive to raising $[Ca^{2+}]_i$ and thus seem to be outfitted with a β - subunit. Whether BK channels in glioma cell lines generally are outfitted with a β-subunit is unknown so far. In a study comparable to ours the half maximal activation of BK channels in STTG–1 glioma cells was measured [4]. The value was + 65mV at 10 nM $[Ca^{2+}]$ and + 12 mV at 1 µM $[Ca^{2+}]$ while in 1321N1 cells we found half maximal activation to occur at +241 mV at 10 nM Ca^{2+}] and + 68 mV at 1 µM $[Ca^{2+}]$. Therefore there seems to be a great variability in the Ca^{2+} sensitivity between different glioma cell lines. It has been shown that BK channels are active in glioma cells stimulated with acetylcholine [46] and bradykinin [4] both factors which induce a Ca^{2+} influx [46] [130]. Naturally the more sensitive glioma cells are to increases in $[Ca^{2+}]_i$, the more sensitive they are also to stimuli that induce a Ca^{2+} influx such as many other growth factors do. Finally if a modification in the β - subunit shifts the channels G-V relation along the voltage axis to the left then also unstimulated cells might display BK channel activity at the resting membrane potential. It would be interesting whether an increased Ca^{2+} sensitivity of BK channels achieved by the introduction of a modified β - subunit correlates with malignant behavior of glioma cells.

The Ca^{2+} sensitivity of BK channels is relatively low in any case since $[Ca^{2+}]_i$ has to be elevated to concentrations between 100 nM and 1 µM to activate them. It is noteworthy however that activation of BK channels does not require a marked elevation of the total cytosolic $[Ca^{2+}]$. In fact it is sufficient to raise the submembrane $[Ca^{2+}]_i$ near the β-subunit of the channels [3]. Ca^{2+} can be supplied from the extracellular space via Ca^{2+} channels closely colocalized with BK channels [131]. In chicken sympathic and parasympathic neurons K_{Ca} channels are functionally dependent to either L - or N – type Ca^{2+} channels. This was experimentally prooven with specific Ca^{2+} channel blockers which could diminish IK_{Ca} elicited by depolarizing

voltage steps because they blocked the concomitant Ca^{2+} influx through either L - or N – type Ca^{2+} channels required to gate IK_{Ca} [132]. *In vivo* in such excitable cells depolarization during the action potential causes a Ca^{2+} influx through voltage gated Ca^{2+} channels. Concentration peaks that are much higher than the measured average total cell $[Ca^{2+}]_i$ may occur in close proximity to BK channels [133-136]. BK channels are thus activated and cause the after hyperpolarization following the action potential. In nonexcitable cells it is less clear which Ca^{2+} influx pathways form a functional unit with BK channels. In glioma cells a depolarizing stimulus, which opens voltage, gated Ca^{2+} channels and thus activates BK channels might be a growth – factor such as elevated $[K^+]_e$. For instance T – Type Ca^{2+} channels and BK channels interact in mediating growth factor induced proliferation in Müller glial cells [43]. The Ca^{2+} influx pathways that provide the Ca^{2+} necessary for BK channel gating in glioma cells are however still unknown.

5.2.2 Pharmacology of BK channels

BK channels were identified by blockade with the specific blocker IBTX and 1 mM TEA which at this concentration (< 1 mM) is known to be a specific BK channel blocker [109] [109, 110, 112]. K^+ channels are divided into such with high TEA sensitivity (Kd < 1 mM), moderate TEA (Kd = 10 mM) sensitivity and low TEA sensitivity (Kd > 50 mM). However there are also TEA insensitive BK channels. [137] [138]. These differences in TEA senstivity can be explained on a molecular basis. Voltage gated K^+ channels are composed of six transmembrane segments (S1 to S5). TEA, CHTX and IBTX have the same binding site which lies in the S5 – S6 linker. This region is built from 40 amino acids including two basic and four acidic ones [139]. By modifications in the aminoacid residues in this region the TEA and CHTX sensitivity can be changed dramatically. Changes at the residues 449 to 451 have the most marked effect [140, 141]. In glial cells low concentrations of TEA had no effect on inward rectifier, A-type, or delayed rectifier channels but effectively blocked BK channels [112]. We therefore used 1 mM TEA as a specific BK channel blocker in 1321N1 glioma cells. The IC_{50} for TEA was determined to be 0.19 mM in our experiments. A similar value was observed in cells from other gliomas [4], meningiomas [86] and GH3 pituitary cells [109] also at low intracellular $[Ca^{2+}]$ of 10 or 20 nM. An

increase of TEA to 10 mM did not further reduce the current amplitude. The amount of current inhibition in our experiments caused by 1 mM TEA lay in the range of that observed for 100 nM IBTX indicating no significant difference between 1mM TEA and 100 nM IBTX potency at any voltage.

In summary, the electrophysiological recordings and pharmacological experiments identified outward currents in 1321N1 cells to be mediated by BK channels. In addition the experiments confirmed that TEA at concentrations of 1 mM is a specific blocker of BK channels and can be used as a cheap tool for screening BK channel activity.

5.2.3 BK channels in 1321N1 human astrocytoma cells are active under physiological conditions

The prerequisite for a physiological function of BK channels is a significant open probability at physiological resting membrane potentials. Generally, BK channels have a low open probability in resting cells, but once the cell becomes depolarized the channels are activated to then dominate the membrane potential [142]. Only few channels of this type need to be active to mediate large K^+ fluxes and thus it has profound effects on membrane potential oscillations [142].

Because whole cell recordings require unphysiologically strong depolarisations for BK channels to open even with 1 µM $[Ca^{2+}]i$ one could assume that these channels are not active in resting 1321N1 human glioma cells. However the conduction voltage relation in **Figure 6D** shows BK channel activity at physiological membrane potentials with 1 µM $[Ca^{2+}]_i$. To confirm this we chose a method that allowed testing BK channel activity at physiological membrane potentials at various $[Ca^{2+}]_i$ with better resolution than whole cell recordings.

Therefore we use an excised patch model to simulate BK channel activity around the physiological resting membrane potential. Physiological KCl concentrations were reversed so there were 130 mM KCl outside and 13 mM KCl inside. Thus large BK channel mediated inwardly rectifying K^+ currents could be elicited while the damped potential was maintained at – 20 mV and the Ca^{2+} concentration in the bath was varied. Our results demonstrate that there is marked BK channel activity at the resting membrane potential when physiologic Ca^{2+} concentrations are present.

The increase in open probability between 100 nM and 1 µM Ca^{2+} was significant. However the interpretation of excised patch experiments to determine BK channel activity around the typical resting membrane potential is difficult because no experimental condition can adequately simulate the balance between activating and inhibitory factors *in vivo*. What makes excised patch recordings especially problematic to interpret is the finding that BK channels are modulated by the cytoskeleton. Cytoskeletal alterations due to a proteolytic agent had an activating influence on BK channels and caused a moderate rise in P_o, but patch excision and channel isolation from the natural environment provoked a strong increase in P_o [84]. A study by Kraft et al. [86] on BK channels in human meningeoma cells also suggests the same. The application of cytochalasin D a drug that disassembles the cytoskeleton increased P_o of BK channels in cell-attached patches very rapidly [86].

Several studies proove that in addition to being voltage and Ca^{2+} sensitive BK channels are activated through many alternative intracellular signalling pathways. Investigations have identified activation by channel protein phosphorylation. When studying the effect of ethanol on BK channels in GH3 pituitary cells it was observed that ethanol significantly increased mean channel open time and channel open probability. This effect was blocked in the presence of protein kinase A (PKA) inhibitors suggesting that the effect of ethanol on BK channels is mediated by PKA stimulation and phosphorylation of the channels [143]. Recently it was found that protein kinases A and C are opponents in modulating glial BK channels. Stimulating the protein kinase A increased the open probability whereas exposure to an activator of protein kinase C strongly reduced the channel activity in Müller glial cells [90]. Moreover an activation of protein kinase C inhibited BK channels in rat pituitary tumour cells [89]. This indicates that BK channels are the effectors of different signaling pathways [90]. However the regulation of BK channels by protein kinases depends on the cell context since in transfected cells the expressed BK channel is not regulated by protein kinase activity [93]. Moreover somatostatin stimulates BK channels in rat pituitary tumor cells through lipoxygenase metabolites of arachidonic acid, which is additional evidence that protein phosphatases directly regulate K^+ channel activity [144].

Other studies indicate that BK channels are subject to redox regulation [92, 115] because chloramine-T a substance that oxidizes methionin shifted the steady-state macroscopic conductance to a more positive direction. Regulation of the BK channel by methionine oxidation may be an important link between the membrane potential and metabolism [92]. The stimulation of the activity of BK channels by intracellular protons was also recently observed in smooth muscle cells. Alkalinisation of the cytosol markedly shifted the channels' IV relation to the left while an increase of the intracellular proton concentration inhibited this channel [91]. In excised patch experiments these intracellular factors are eliminated.

Generally in patch clamp experiments many channel regulating factors like those mentioned above present *in vivo* might be absent. Our excised patch experiments, however, are nonetheless a reasonable model for channel activity in 1321N1 cells at physiological membrane potentials. The most important conclusion that can be drawn from this model is the fact that BK channel activation seems to not necessarily require a marked depolarization of the membrane potential. Instead it is sufficient to provide a $[Ca^{2+}]_i$ between 100 nM and 1 µM to the inner face of the membrane to activate these channels. This disputes the theory that voltage is the prime activator of BK channels [83] and is more in line with a study that found Ca^{2+} to be the stronger activator at physiological membrane potentials: DiChiara et al. [145] observed that the activation kinetics of *dslo* (drosophila) BK channels were strongly influenced by the $[Ca^{2+}]_i$, but were only minimally affected by membrane voltage. Current activation kinetics increased more than 60-fold in response to increases of $[Ca^{2+}]_i$ in the range 0.6 - 400 µM, but increase less than 2-fold by voltage changes from -60 to +80 mV. The *hslo* (human) BK channels were somewhat more sensitive to voltage but Ca^{2+} remained the more potent activator [145]. An ion channel that would require very strong membrane depolarisations for its activation would not likely have a physiologic function in nonexcitable cells. Indeed our whole cell experiments with 10 nM $[Ca^{2+}]$ would rather suggest that BK channels are not active at physiological membrane potentials in 1321N1 cells while with 1 µM there seems to be some activation. The excised patch experiments however demonstrated channel activity at physiological membrane potentials. A physiological role of BK channels in 1321N1 cells is therefore conceivable.

5.3 BK channels and their role in in cell proliferation

Little work has been done to investigate the possible role of BK channels in neoplastic cells. Here, we were able to demonstrate in 1321N1 human astrocytoma cells that under certain conditions they play a crucial role for proliferation. Several growth factors may activate BK channels through one of the mechanisms mentioned above. BK channel activation is a signal, which induces proliferation. Since this channel is common to glioma cells it is striking that it seems to serve one specific function, which might be the promotion of proliferation induced by a certain category of growth factors. However this study also shows that there is a component of proliferation, which is independent of BK channel activity.

5.3.2 Blockade of BK channels influences proliferation only under particular conditions

If BK channel activation is causative for cell cycle progression, then it should be possible to block proliferation with specific BK channel blockers. Earlier studies have failed to demonstrate a growth inhibitory effect of IBTX in various different cell types, which express BK channels. Wondergem et al. [146] showed that neither IBTX nor CHTX could inhibit the growth of human bladder tumor cells (HBT-cells). For the proliferation of astrocytoma cells, neuroblastoma cells and meningeoma cells other studies also failed to show an inhibitory effect of IBTX and CHTX [4, 31, 32, 48, 86] [94]. On the other hand there are studies, which show that BK channel blockade inhibits proliferation. However this is the case only when they studied a component of proliferation that was stimulated by certain growth factors [35, 42, 43, 51, 112]. We were able to demonstrate that glioma cell proliferation could be blocked by specific BK channel inhibitors when 1321N1 cells were growth stimulated by an elevated $[K^+]_e$. Elevating the K+ concentration in the growth media from 5 to 20 mM resulted in an increase in the cell number of 20 %. after 5 days. BK channel inhibitors could block this K^+-induced proliferation only.

K^+-induced proliferation

Analogous evidence that elevating the K^+ concentration can enhance proliferation has been gathered for retinal glial cells (Müller cells) [147], astrocytes [148] and immature cerebellar granule cells [149]. In these studies the K^+ concentration was not higher than 20 mM. On the other hand at very high concentrations (> 60 mM) $[K^+]_o$ has a growth inhibitory effect [150]. We have performed experiments with 20, 50 and 100 mM $[K^+]_e$. We observed that prolonged elevation of $[K^+]_e$ promotes proliferation at $[K^+]_e$ between 5 and 20 mM but significantly inhibits it at concentrations above 50 mM. This is in accordance with the work of Canady et al. [150] who found a decreased DNA synthesis in cultured glial cells after long term exposure to $[K^+]_e$ above 60 mM. Short term exposure to 60 mM $[K^+]_e$ however stimulates glial cell proliferation [148]. At moderately elevated $[K^+]_e$ (20 mM) glial cell proliferation seems to be optimal in long term experiments as was also shown also in other studies [43, 51]. This dose response relation is valuable in guiding future experiments.

That moderately elevated $[K^+]_e$ can enhances brain tumor cell proliferation via the activation of a specific ion channel common to glioma cells is a new and exciting finding. Moreover, this finding is of particular relevance for CNS neoplasms in vivo. One might speculate that in malignant tumors an extensive necrosis of part of the tumor cells and neurons generates an increase of $[K^+]_e$ [151] which in turn promotes the proliferation of neoplastic cells. Unfortunately clinical data which proove this are lacking. However normal neuronal activity already causes $[K^+]_e$ to rise from a baseline of 3 mM to levels over 10 mM [152, 153]. In epileptic tissue elevated $[K^+]_e$ was detected during ictal activity [154] and during anoxia and spreading depression a $[K^+]_e$ of 40 – 100 mM has been measured [151, 155-158]. Therefore neoplastic glial which come in close contact with normal neuronal tissue due to their marked tendency to spread within the brain will also likely be stimulated by elevated $[K^+]_e$ released in their surroundings. It is thus conceivable that the enhanced growth of astrocytoma cells under conditions of elevated $[K^+]_e$ are the rule rather than the exception in vivo and that previous studies on K^+ channel blockers and proliferation failed to consider this possibility. It is furthermore conceivable that in vivo the K^+-induced component of proliferation might even be greater than the 21% found in our

study since the rate of tumor cell proliferation in vitro depended mainly on the supply with high concentrations of FCS (10 %) unlikely to be seen in vivo.

A role for BK channels for K^+-dependent proliferation was recently found for cultured Müller glial cells [43]. Like in our study the authors found an inhibitory effect of IBTX (100 nM) and TEA (1 mM) on DNA synthesis only at elevated [K^+] (25 mM) whereas no effect was seen at a K^+ concentration of 5.8 mM. A former study by Reichenbach et al. [51] yielded essentially the same results. Both studies are well in accordance with our findings of an effective block of 1321N1 astrocytoma cell proliferation by 1 mM TEA and 100 nM IBTX under depolarizing conditions with 20 mM $[K^+]_o$ in the culture medium.

Growth factor-stimulated proliferation

High $[K^+]_e$ might just be one example of many factors that induce proliferation by activating BK channels: many growth factors lead to membrane depolarization and $[Ca^{2+}]_i$ increase may thus activate BK channels. Such was shown for human astrocytoma cells following stimulation with muscarine that is a potent mitogene [46]. To identify the mechanisms by which growth factors enhance brain tumor growth is an important field of research because braintumors will be exposed to a multitude of growthfactors in their environment [10].

Inhibition of **growth factor-stimulated proliferation** by BK channel blockers has been demonstrated in several studies. Wiecha et al. [42] showed that IBTX in human vascular endothelial cells inhibited proliferation stimulated by the basic fibroblast growth factor. In addition, Vaur et al. [35] demonstrated that TEA in concentrations of 1 – 4 mM blocked proliferation of pituitary tumor cells by a specific cell cycle-block during the G1/S transition. Because only BK channels are sensitive to TEA in this concentration range while IK and SK channels require much higher doses of TEA [159] [160] the inhibition of proliferation was attributed to specific BK channel block. In Müller (retinal) glial cells iberiotioxin and TEA 1 mM were found to block EGF-induced proliferation as well as proliferation induced by high $[K^+]_e$ [43]. However there is also one study on this topic that presented conflicting data. VEGF-A (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) activated BK channels in HUVEC (human vascular endothelial cells) and strongly stimulated their proliferation. However IBTX did not inhibit this induction of proliferation indicating that

BK channel activity is not involved in VEGF-A- or bFGF-induced HUVEC proliferation [94]. The latter study must be interpreted with caution since only one BK channel blocker (IBTX) was used in the proliferation experiments. Our investigations of BK channel pharmacology revealed that IBTX and TEA (1 mM) blocked the outward currents in 1321N1 cells by 50 – 70 % only. The pool of channels not blocked by IBTX in HUVEC therefore might have still been large enough to account for the full proliferative response to VEGF-A- or bFGF. Therefore if one blocker does not produce an effect other blockers such as TEA or paxiline should also be tested. For a review on the pitfalls of testing the antiproliferative response of K^+–channel blockers see [25].

Our results of K^+-dependent growth inhibition in gliomas by BK channel blockers, which are in accordance with the studies on growth factor-dependent proliferation in other cells as mentioned above, strongly support a link between mitogen signalling and BK channels in human gliomas. We speculate that BK channels – although not crucial for basal serum dependent proliferation – are key players in proliferative activity, which is stimulated by a certain class of mitogens. These mitogens can activate BK channels through one of the many pathways discussed above.

5.3.3 Possible mechanisms of involvement of BK channels in cell proliferation

From the available studies the following hypothesis explaining the mechanism of the involvement of BK channels in cell proliferation can be constructed:

Following membrane depolarization by certain growth factors voltage gated Ca^{2+} channels open and an initial Ca^{2+} spike occurs in close proximity to BK channels. This together with membrane depolarization opens BK channels and thus increases gK. The resulting hyperpolarization of the membrane potential constitutes the driving force for further Ca^{2+} entry from the extracellular space and moreover causes a liberation of Ca^{2+} from intracellular stores. Because voltage gated Ca^{2+} channels close when the membrane becomes hyperpolarized the Ca^{2+} influx from the extracellular space must be sustained by voltage independent pathways. Recently non-selective cation channels (NSCC) have been impicated as the mediators of such a prolonged Ca^{2+} influx. The resulting Ca^{2+} spike is the trigger mechanism for the progression of the cell through the G1 – S START point. The following data

support the hypothesis which was just presented: Two studies confirmed the activation of voltage gated Ca^{2+} channels following the exposure of cells to elevated $[K^+]_e$: In cerebellar granule cells elevated $[K^+]_e$ caused an initial Ca^{2+} influx through L – type Ca^{2+} channels. The increase in DNA synthesis elevated $[K^+]_e$ induced in these cells could be reduced by blockers of the L – type Ca^{2+} channel [149]. In glial cells this initial Ca^{2+} influx seems occur through T – type Ca^{2+} – channels. The growth stimulatory effect of high $[K^+]_e$ on cultured retinal glial cells [43] could be reversed with with a Ca^{2+} channel blocker selective for T–type Ca^{2+} channels (flunarizine). BK channels therefore are gated by submembrane calcium peaks, which are due to a Ca^{2+} influx through voltage-gated Ca^{2+} channels closely colocalized with BK channels. Additionally BK channels are activated directly by elevated $[K^+]_e$. As pointed out by Ransom et al. (2001) [4] BK channel activity is proportional to the square root of $[K^+]_e$. In two other studies it was observed that the single channel conductance increased dramatically with elevating K^+ in the bath solution [117, 161, 162]. The liberation of mobilization of Ca^{2+} from intracellular sources following growth factor stimulation has also been documented. Catlin et al. [163] studied the Ca^{2+} response of 1321N1 cells to carbachol which is mediated by the M3 subtype of muscarinic receptors [163]. The authors observed an initial Ca^{2+} spike which was due to Ca^{2+} release from intracellular Ca^{2+} stores followed by a sustained elevation and oscillations which were dependent on Ca^{2+} influx from the extracellular space. The pathways for such a delayed and sustained Ca^{2+} influx seem to be voltage independent channels such as NSCC [27, 164]. Recently the Ca^{2+} channels involved in endothelin-1 (ET-1) induced mitogenesis of C6 glioma cells were identified to be NSCC [27] **(Figure 14)**.

5.3.4 BK channels and their role in cell migration

For a glioma cell to spread within the brain parenchyma it requires among others two important abitities. It must shrink enormously to permeate the narrow spaces within the neuropil texture or to move along blood vessels. Furthermore it must have a mechanism that propulses the cellbody forward. The cells ability to permeate narrow spaces can be studied with the Transwell assay while the cells ability to locomote in

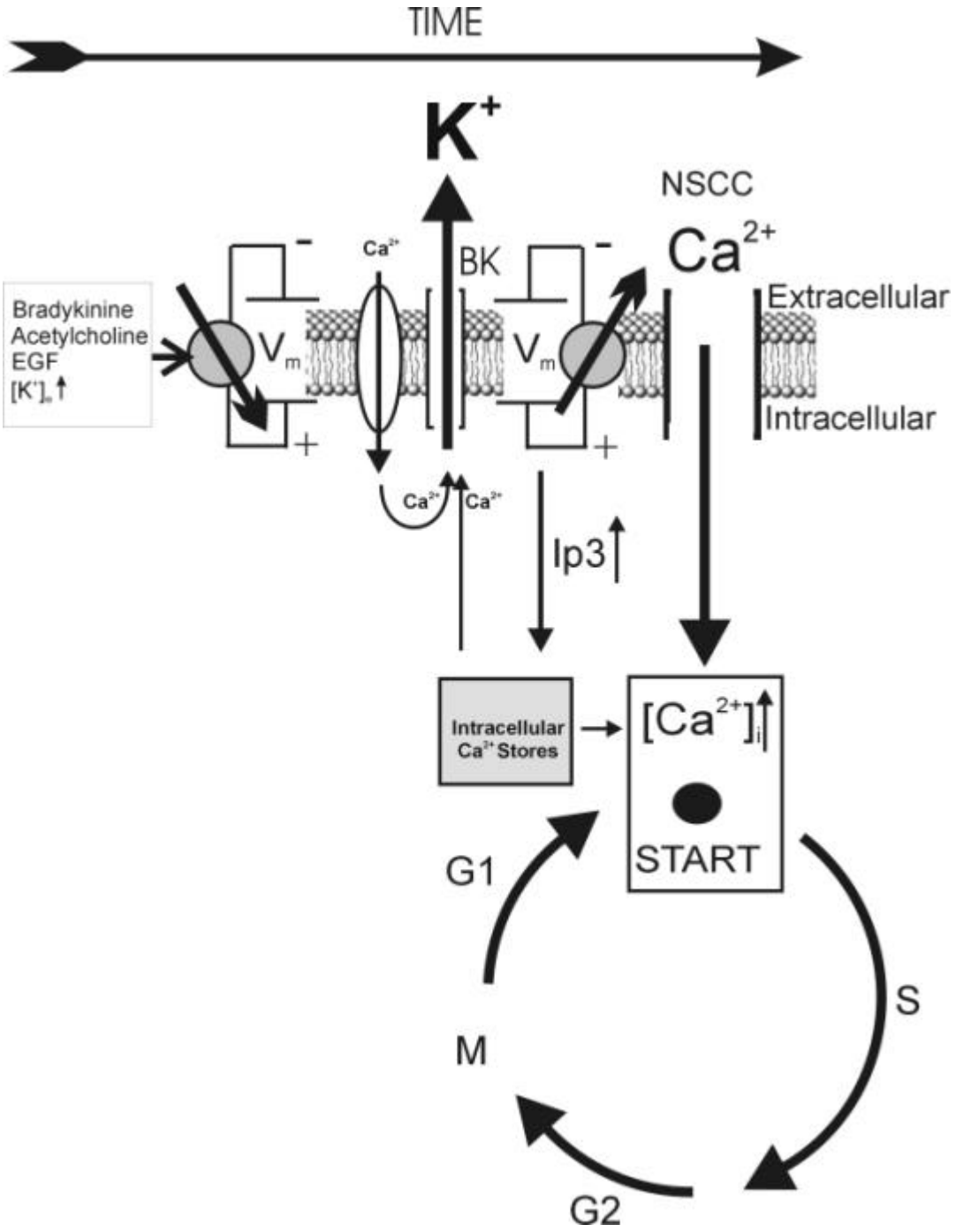

Figure 14 – The role of K^+ channels in cell proliferation.
Following membrane depolarization by growth factors or elevated $[K^+]_e$ voltage gated Ca^{2+} channels open and account for the Ca^{2+} influx required for BK channel gating. Membrane depolarization and external K^+ ions further facilitate BK channel gating. The rise in K^+ conductance causes the membrane to hyperpolarize. This constitutes the driving force for Ca^{2+} influx through NSCC (nonselective cation channels). The hyperpolarized membrane potential also causes a rise in IP_3 production and thus a release of Ca^{2+} from intracellular stores. A peak of $[Ca^{2+}]_i$ is the trigger for the progression of the cell cycle through the START controll point between G1 an S phase.
(Modified according to [184]).

the plane and the velocity with which this occurs can be studied with a timelapse setup.

In the following paragraph the term migration connotes both the ability to shrink and the ability to move in a plane and through holes into the lower compartment of the Transwell chamber while the term motility connotes only the ability to move in a plane as studied with the timelapse technique although in other studies this term has occasionally been used to define oscillations of the cell membrane without cell locomotion observed for instance with an atomic force microscope. The term migrational velocity refers to the speed of cell locomotion measured with the timelapse technique and thus without spational restraints. It is also possible to study tumor invasion with the Transwell assay. For this purpose the filter membranes are coated with a matrix protein, which the cells then will digest if they are invasive. Strictly speaking therefore tumor invasion was not studied in our transwell experiments although some studies have referred to simple transwell permeation as invasion.

This study examined the role of BK channels in the physiology of migration. It has been recently proposed that glioma cells upregulate chloride channels to secrete chloride accompanied by water thus shrinking to a size that allows them to permeate the narrow spaces in the brain [72]. The latter investigation looked at the effect of ion channel blockers on transwell permeation and glioma invasion into brain sclices using the U251MG human glioma cell line. Significant inhibitory effects for TEA 1 mM, CLTX 1 µM and IBTX 10 nM was observed. With TEA and CLTX a 50 % effect was seen while IBTX could inhibit invasion by 20 %. It is doubtful that the study really prooves a role for chloride channels in the migration of glioma cells as recently CLTX was shown to not have any effect on chloride channels [73]. Moreover, in that study IBTX was applied in a dose that is too low to effectively block all BK channels but nevertheless it had a profound effect. For all these reasons we suspect that the three blockers used in the study by Soroceanu et al. (1998) [72] excerted their effect by blocking BK channels. It was therefore appropriate to again test the potential of TEA and 100 nM IBTX on glioma migration under the assumption that volume regulatory mechanisms involve BK channels in these cells.

We applied two techniques: the transwell assay to study glioma migration through narrow spaces and time-lapse videomicroscopy to study migrational velocity. First, we tested two specific blockers of BK channels, IBTX and 1 mM TEA. They both had neither a significant anti-migratory effect nor did they slow migrational velocity. TEA started to be effective only at concentrations higher than 5 mM. Soronaceanu et al. (1998) [72] also found no effect of BK channel blockers on the migrational velocity of glioma cells (using the scrap motility assay) however when migration through the narrow spaces of transwell membranes was studied IBTX and TEA were effective. TEA in a concentration of 1 mM was however able to halt glioma invasion into rat brain slices [72].

There are several possible explanations for the discrepancy of our findings to those of the latter study. The main methodical difference in the transwell experiments between the two studies is that we used 10 % FCS in the migration assay buffer while the quoted authors studied migration under serumfree conditions. In our preliminary experiments we found 1321N1 cells to not migrate through transwell filters under serumfree conditions and therefore we choose to study migration in the presence of serum. It is unlikely that IBTX or TEA were deactivated in the presence of serum since the two blockers were able to effectively block K^+-dependent proliferation in other experiments.

The most likely explanation for the different outcome of the two studies is that there are two different mechanisms underlying invasion one being BK channel-dependent in the presence of low concentrations of FCS and one being independent of BK channel activity in the presence of high concentrations of FCS. Indeed the migration of human astrocytoma cells (studying astrocytoma cells from the two human cell lines U-87MG and A172) was experimentally dissected into four separate components which rely on Ca^{2+} oscillations and the presence of serum to a varying degree: (i) a Ca^{2+}-dependent / serum-independent component, (ii) a Ca^{2+}-independent / serum-dependent component, (iii) a Ca^{2+}- and serum-independent component as well as (iv) a Ca^{2+}-dependent and serum-dependent component [165]. One of the two cell lines in this study (the U-87MG human glioma cell line) lacked the Ca^{2+}-dependent and serum-independent component. Thus considering the great heterogeneity among astrocytoma cell lines there might be tumor cells which can migrate without depending on intracellular Ca^{2+} oscillations. In those cells which

depend on them to move Ca^{2+} oscillations might trigger a cyclic activity of K_{Ca} channels including BK channels leading to cyclic cell shrinkage and swelling accounting thus for the propulsion of the cell. The interplay between K_{Ca} channels, $[Ca^{2+}]_i$ and the cytoskeletton has been well explained in a review [53]. Interfering with this complexly orchestrated machinery of cell crawling at the level of the K_{Ca} channels was shown to halt MDCK cell migration [55].
Possibly in our experiments the Ca^{2+}-independent but serum-dependent component outweighed all other components of migration. When lower concentrations of FCS are applied then there might be a stronger weight on Ca^{2+}- and BK channel-dependent migration. This hypothesis should be investigated in a followup study. It has to be remarked that 10 % FCS are not likely to be present in the brain interstitium and, analogous to experiments on glioma proliferation, investigations under more physiologic conditions should be undertaken.

An alternative explanation for the ineffectivity of BK channel blockers on 1321N1 cell migration is that our cells have a large functional BK channel reserve which was not completely and constantly blocked in our experiments and only a small fraction of BK channes need to be active to maintain normal cell function. Only brief bursts of BK channels are necessary to expel enough K^+ for a profound volume decrease. For MDCK cells it was calculated that per burst of high K^+ channel activity as much as 20 % of the cells´ total K^+ can be expelled [166]. Moreover the rate of migration was proportional to the K^+ channel activity [53]. Our investigations of BK channel pharmacology revealed that IBTX and TEA (1 mM) blocked the outward currents in 1321N1 cells by 50 – 70 %. The pool of channels not blocked might still be large enough to account for intact volume regulation. The spare receptor theory predicts an increase in the IC_{50} for migration relative to K^+ current inhibition: the concentration of an antagonist required to inhibit a response by 50 % (EC_{50}) will be greater than the Kd of the antagonist for its receptor if many spare receptors are present [167]. Thus it can be explained why TEA started to have an effect on both migrational velocity and transwell permeation only in concentrations of 5 mM.

Finally at concentrations above 1 mM TEA might also have other targets than just BK channels. Because TEA can enter cells nonspecific inhibitory actions cannot be excluded. When studying lymphocyte proliferation, Schell et al. (1987) [168] found

that TEA excerted its antiproliferative effect by inhibiting the uptake of essential cell metabolites such as amino acids. The inhibitory effect of 5 mM TEA on 1321N1 cell migration should therefore be interpreted with great caution. Since with paxilline recently another very potent, highly specific BK channel inhibitor has become available [169], we would like to suggest that it is worthwhile to repeat experiments on invasion and migration with this drug.

Together with our data on BK channel involvement in mitogene-induced proliferation we like to propose the following theory: BK channel activation induces a proliferative response in 1321N1 glioma cells and thus halts migration ('go *or* grow'). As pointed out in the introduction, the theory of a dichotomy of migration and proliferation is well established [101], i.e., that cells either crawl *or* divide. Thus the theory of a dichotomy of proliferation and migration was proven for the function of a single type of ion channel (see also [46]).

5.4 Effect of EGF on BK channels

Studies on several cell types including glioma cells reported that EGF is a potent mitogen [14, 107, 170-174] and studies, which linked it to **motility and invasion** promoting effects [9, 12-14, 98, 103, 104, 106, 107, 171, 172, 175, 176].

In our study we looked at the effect of EGF on BK channels because there was indications that EGF signalling might involve the modulation of ion channels – especially K_{Ca} channels [44, 102, 173, 177].

Direct evidence that BK channels are inducible by EGF stemmed from the observation that induction of a K_{Ca} channels in murine fibroblast cell lines depends on induction in nontransformed cells by EGF and PDGF and K^+ channel blockers are able to inhibit cell proliferation [178] .
We here showed that, after a 6 day exposure to EGF, a more than twofold increase in outward K^+ currents could be observed indicating upregulation of K^+ channels through EGF. We could furthermore show that EGF significantly depolarized 1321N1 cells after 6 days in culture. A previous study on osteoblasts also found a

depolarization of cells after prolonged (6 day) exposure to EGF [179]. This effect could be reversed with 10 µM nifedipine indicating the involvement of Ca^{2+} - dependent processes in the EGF response [179]. This is in accordance with data that showed that EGF increased Ca^{2+} influx [174, 180, 181], which may result in subsequent induction of DNA synthesis. Indeed, in one study on Müller glial cells it was observed that EGF induced DNA synthesis. Moreover measurements of $[Ca^{2+}]_i$ revealed that EGF induced a prolonged elevation of $[Ca^{2+}]_i$ in these cells. Both effects were fully reversed when either IBTX, 1mM TEA or flunarizine or a specific blocker of the T-Type Ca^{2+} channel was added. It was therefore concluded that BK channels regulate the EGF stimulated DNA synthesis of Müller cells via feedback regulation of Ca^{2+} entry [43]. On the other hand as already mentioned a study on the effect of VEGF-A (vascular endothelial growth factor) and bFGF (basic fibroblast growth factor) on HUVEC (human vascular endothelial cells) revealed that both growth factors activated BK channels and at the same time strongly stimulated proliferation. However IBTX did not inhibit this induction of proliferation indicating that BK channel activity is not causative for VEGF-A- or bFGF-induced HUVEC proliferation [94]. As also pointed out already the latter study must be interpreted with caution because only IBTX as a BK channel blocker was tested.

The exact mechanism by which $[Ca^{2+}]_i$ is increased in the context of EGF-induced enhanced DNA synthesis is however largely undetermined. Activation of BK channels which accounts for a membrane hyperpolarization is one likely to be responsible for the Ca^{2+} influx. As pointed out earlier, membrane hyperpolariszation augments the electrochemical driving force for Ca^{2+} and leads to a Ca^{2+} influx through voltage-independent pathways such as NSCC [182]. EGF-induced rapid hyperpolarisation followed by a constantly hyperpolarized membrane potential within 30 minutes of its application has been shown, for instance, in human fibroblasts and human carcinoma cells [44, 102]. This hyperpolarization was associated with a steep rise in $[Ca^{2+}]_i$. Coapplication of the K^+ channel blocker quinidine did inhibit the hyperpolarization response. Thus it was concluded that an initial event following EGF receptor stimulation was an activation of K_{Ca} channels [102]. In another study that described growth factor signalling in fibroblasts the application of PDGF or FGF induced, after a lag (0.5-1 min), a $[Ca^{2+}]_i$ increase composed of an initial, slow peak, sustained primarily by intracellular Ca^{2+} release followed by a plateau, sustained by Ca^{2+} influx

from the medium. The $[Ca^{2+}]_i$ changes were paralleled by plasma membrane hyperpolarization mainly due to the activation of a K^+ efflux through K_{Ca} channels. Our data indicate that EGF increases gK in 1321N1 cells. Initially this will hyperpolarize the cells. Since this hyperpolarization causes a sustained Ca^{2+} influx the cell can enter the cell cycle. The total membrane depolarization caused by long therm exposure to EGF might be due to other changes in the cells metabolism or its membrane properties.

6 Conclusions

Electrophysiological recordings and pharmacological experiments identified depolarization induced outward currents in 1321N1 cells to be mediated by BK channels. In addition the experiments confirmed that TEA at concentrations of 1 mM is a specific blocker of BK channels and can be used as a cheap tool for screening BK channel activity. With excised patch recordings we demonstrate that there is marked BK channel activity at the resting membrane potential when physiologic Ca^{2+} concentrations are present. The increase in open probability between 100 nM and 1 µM Ca^{2+} was significant. A significant open probability at physiological resting membrane potentials is the prerequisite for a physiological function of BK channels. The functional significance of BK channels for the disposition of glioma cells to migrate and proliferate was therefore studied.

In a first set of experiments study examined the role of BK channels in the physiology of migration with transwell assays and with timelapse videomicroscopy. We tested two specific blockers of BK channels, IBTX and 1 mM TEA. They both had neither a significant anti-migratory effect nor did they slow migrational velocity. TEA started to be effective only at concentrations higher than 5 mM. Little attention has been so far paid to the adaptative mechanisms that enable glioma cells to reduce their volume and migrate along the narrow gaps within the neuropil. In glioma cells the regulatory volume decrease is probably a consequence of mainly K^+ and Cl^- efflux through ion channels. To date however only few studies exist on the role of ion channels in glioma migration but from the results of our study it can be deduced that 1321N1 cell migration does not rely on BK channel function. However this does not exclude that other glioma cell lines employ BK channels to migrate.

In a second set of experiments we were able to demonstrate that glioma cell proliferation could be blocked by specific BK channel inhibitors when 1321N1 cells were growth stimulated by an elevated $[K^+]_e$. Elevating the K^+ concentration in the growth media from 5 to 20 mM resulted in an increase in the cell number of 20 %. Only this $[K^+]_e$ induced proliferation could be blocked by specific BK channel blockers. This finding is of particular relevance for CNS neoplasms in vivo. One might speculate that in malignant tumors an extensive necrosis of part of the tumor cells and neurons generates an increase of $[K^+]_e$ [151] which in turn promotes the proliferation of neoplastic cells. Unfortunately clinical data which proove this are

lacking. However normal neuronal activity already causes $[K^+]_e$ to rise from a baseline of 3 mM to levels over 10 mM [152, 153]. In epileptic tissue elevated $[K^+]_e$ was detected during ictal activity [154] and during anoxia and spreading depression a $[K^+]_e$ of 40 – 100 mM has been measured [151, 155-158]. Therefore neoplastic glial which come in close contact with normal neuronal tissue due to their marked tendency to spread within the brain will also likely be stimulated by elevated $[K^+]_e$ released in their surroundings. It is thus conceivable that the enhanced growth of astrocytoma cells under conditions of elevated $[K^+]_e$ are the rule rather than the exception in vivo and that previous studies on K^+ channel blockers and proliferation failed to consider this possibility.

In a final set of experiments we here showed that, after a 6 day exposure to EGF, a more than twofold increase in outward K^+ currents could be observed indicating upregulation of K^+ channels through EGF. We however were not able to conclusively demonstrate that this increase of gK^+ is due to BK channel upregulation. We could furthermore show that EGF significantly depolarized 1321N1 cells after 6 days in culture. It is however still undetermined whether EGF stimulates the proliferation of 1321N1 cells.

In conclusion our data suggest that BK channel activation in 1321N1 cells is a crucial step in proliferation induced by certain mitogenes. The initial Ca^{2+} signal for the activation of BK channels could be derived from intracellular stores or through an initial short activation of voltage gated Ca^{2+} channels by a growth factor. The hyperpolarization that follows an increase in K^+ conductance may be the driving force for further Ca^{2+} entry. The pathways for this delayed massive Ca^{2+} entry could be NSCC as it was shown for C6 glioma cells. The Ca^{2+} spike could be the actual trigger signal for the cell to enter the cell cycle. We therfore like to propose the following hypothesis: BK channel activation induces a proliferative response in 1321N1 glioma cells and thus halts migration (‘go *or* grow’). As pointed out in the introduction, the theory of a dichotomy of migration and proliferation is well established [101], i.e., that cells either crawl *or* divide. Thus our findings indicate that the theory of a dichotomy of proliferation and migration holds true for the function of a single type of ion channel in 1321N1 astrocytoma cells (see also [46]).

7 Summary

Human glioma cells express a variety of voltage-gated ion channels including Na^+ and K^+ channels [1, 2, 123, 183]. BK channel currents were frequently observed suggesting an ubiquitous expression of these channels in cultured glioma cells [3, 4, 123]. We confirmed the presence of BK channels in 1321N1 cells. The channels we observed in our patch clamp experiments displayed typical features of BK channels combining the properties of voltage– and ligand gated ion channels. BK channels were identified by blockade with the specific blocker IBTX and 1 mM TEA which at this concentration (< 1 mM) is known to be a specific BK channel blocker [51, 159]. Because BK channels require unphysiologically strong depolarisations to open even with 1 µM $[Ca^{2+}]_i$ during whole cell recordings one might assume that these channels are not active in resting 1321N1 human glioma cells. Excised patch recordings however allowed us to demonstrate that BK channels in 1321N1 human astrocytoma cells are active at physiological membrane potentials. A physiological role of BK channels in 1321N1 cells is therefore conceivable.

Despite their stable expression in glioma cells a functional role of BK channels has so far not been identified. However, it is well known from studies with broad-spectrum K^+ channel blockers that other K^+ channels play an essential role during the progression of cells through the G1 phase of the cell cycle [25]. We observed that specific BK channel blockers inhibit proliferation of 1321N1 astrocytoma cells only under particular conditions: Elevating the K^+ concentration in the growth media from 5 to 20 mM resulted in an increase in the cell number of 20 %. after 5 days. BK channel inhibitors could block this K^+-induced proliferation only. We moreover performed experiments with 20, 50 and 100 mM $[K^+]_e$ and observed that prolonged elevation of $[K^+]_e$ promotes proliferation at $[K^+]_e$ between 5 and 20 mM but significantly inhibits it at concentrations above 50 mM. High $[K^+]_e$ might just be one example of many factors that induce proliferation by activating BK channels: many growth factors lead to membrane depolarization and $[Ca^{2+}]_i$ increase may thus activate BK channels. Inhibition of growth factor-stimulated proliferation by BK channel blockers has already been demonstrated in several studies in other cell types [35, 42, 43, 49, 51]. We therefore speculate that BK channels – although not crucial for basal serum dependent proliferation – are key players in proliferative activity, which is stimulated

by a certain class of mitogens. To identify the mechanisms by which growth factors enhance brain tumor growth is an important field of research since brain tumors will be exposed to a multitude of growth factors in their environment [10].

Because ion channels modulators including some specific BK channel blockers were also observed to halt migration of glioma cells [46, 72], we studied the effect of specific BK channel blockers on 1321N1 migration. We found that specific block of BK channels does not influence migration of 1321N1 astrocytoma cells.

Finally we looked at the effect of EGF on BK channels because there was indications that EGF signalling might involve the modulation of ion channels – especially K_{Ca} channels [44, 102, 173, 177]. We here showed that, after a 6 day exposure to EGF, a more than twofold increase in outward K^+ currents could be observed indicating upregulation of K^+ channels through EGF. We could furthermore show that EGF significantly depolarized 1321N1 cells after 6 days in culture.

While it has been established that BK channels are common to almost all human tissue their functional role is only very vaguely characterized [4]. Because these channels in some experimental settings were shown to require strong depolarizations for gating the question arises whether in nonexcitable cells they are at all functional in nonexcitable cells [83]. Furthermore it is unresolved whether these channels display a common cellular function irrespective of the cell type in which they occur, or whether they have specific functions in different cell types. Even if these channels are ubiquitous they might be not functional and redundant in some cell types but essential for proper function or even survival in others. Therefore BK channels might well play an essential functional role in neopastic cells while being an item of peripheral importance in nonneoplastic cells.

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2000 World Congress of Neurology, London

Danksagung

Besonderer Dank gilt Herrn Prof. Stephan Patt ohne dessen umfassende Unterstützung diese Arbeit nicht zustandegekommen wäre. Herr Prof. Patt hat mir wissenschaftliches Denken und Arbeiten beigebracht und mich ermutigt, eigene Ideen zu entwickeln und umzusetzen. Diese Zusammenarbeit hat mich sehr weit vorangebracht.

Herrn Prof. Klaus Benndorf danke ich sehr für seine Bereitschaft, zu jeder Zeit meine Ideen und Experimente zu diskutieren. Nicht zuletzt gebührt ihm auch größte Anerkennung, für die großzügige Bereitstellung einer Fülle von technischen Geräten sowie unserer Laborräume.

Ein herzliches Danke auch an Herrn Dr. Robert Kraft. Unsere zahlreichen offenen und anregenden Diskussionen haben mir sehr geholfen. Leider können sie über die Entfernung nur noch selten stattfinden. Ferner hat Herr Kraft mehrere Messungen mit IBTX beigesteuert und die Dosis – Wirkungskurve für erhöhtes Kalium und Proliferation bestimmt.

Große Hilfe habe ich bei meiner Arbeit auch durch Herrn Christian Bollensdorf erfahren, vor allem bei der Lösung der vielen kleineren und größeren technischen Probleme und durch seine Hilfe bei der Planung der Experimente. Auch er war mir immer ein wertvoller Diskussionspartner.

Auch mit Herrn Lutz Liebmann und Herrn Peter Krause habe ich eng zusammengearbeitet. Herr Krause hat die Timelapse Experimente durchgeführt und Herr Liebmann hat sich um die Funktionsfähigkeit unseres Standes gekümmert.

Engagierte Unterstützung erfuhr ich durch Frau Raabe, Frau Karin Schonknecht und Frau Klupsch bei allen Problemen der Zellzucht und Immunhistochemie.
Bei Michael Haas möchte ich mich für die Fotos von den Transwellmembranen bedanken.

Weiterhin bedanke ich mich noch bei Herrn Dr. Christoph Biskup für viele praktische Tips, manch spannendes Gespräch und nicht zuletzt für seine humorvollen Einlagen. Dem ganzen Benndorf Team gilt meine Annerkennung und Dankbarkeit für die immer angenehme Arbeitsatmosphäre.

Jena im Februar 2002

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9.3 Eidestattliche Erklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist, ich die Dissertation selbst angefertigt habe und alle von mir benutzen Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind, mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. Stephan Patt, Prof. Dr. Klaus Benndorf, Dr. Robert Kraft, Christian Bollensdorff, Peter Krause und Lutz Liebmann, die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für die Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Anhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, den 14.02.02

Daniel Basrai

9.4 Zusammenfassung

Gliomzellen menschlicher Herkunft sind mit einer Reihe verschiedener spannungsabhängiger Ionenkanäle ausgestattet. Besonders BK Kanäle wurden in menschlichen Gliomzellinien regelmäßig beobachtet. Dies legt nahe, daß BK Kanäle auch *in vivo* ein fester Bestandteil solcher Zellen sind. Wir konnten das Vorkommen von BK Kanälen für 1321N1 Zellen bestätigen. Die Kanäle, die wir in unseren Experimenten charakterisierten, wiesen typische Merkmale von BK Kanälen auf : Sie öffneten erst bei Spannungen von mehr als +60 mV, waren ausgesprochen abhängig von $[Ca^{2+}]_i$, und liessen sich mit 100 nM IBTX sowie 1 mM TEA spezifisch blocken. Weil jedoch diese Kanäle eine so ausgeprägte Spannungsabhängigkeit zeigten, lag die Annahme nahe, daß diese Kanäle bei physiologischen Membranpotentialen geschlossen sind und deshalb auch keine physiologische Funktion haben können. Excised patch Experimente belegten jedoch, daß diese Annahme falsch ist. Anhand dieser Experimente, die eine gleichzeitige Variation von $[Ca^{2+}]_i$ und des Membranpotentials erlauben, konnten wir zeigen, daß unter physiologischen Bedingungen diese Kanäle offen sein können. Dies ist die Bedingung für eine Funktion von BK Kanälen in menschlichen Gliomzellinien, welche bisher nicht untersucht worden war.

Wir konnten beobachten, daß durch eine Erhöhung von $[K^+]_e$ im Medium von 5 auf 20 mM die Zellzahl nach 6 Tagen um 20 % zunahm. Diese Zunahme der Zellzahl konnte durch spezifische BK Kanal Blocker verhindert werden. Dieser Befund deutet auf eine Rolle der BK Kanäle bei der durch bestimmte Wachstumsfaktoren ausgelösten Proliferation hin. Der genaue Mechanismus, der der Beteiligung von BK Kanälen bei der Proliferation zugrundeliegt ist noch nicht aufgeklärt.

Die Untersuchungen zur Migration von 1321N1 Zellen ergaben, daß hierbei BK Kanäle keine Rolle zu spielen scheinen.

In einem dritten Teil von Experimenten untersuchten wir noch den Einfluß einer längerdauernden Kultivierung mit 10 ng/ml EGF auf die spannungsabhängigen Kaliumkanäle. Wir konnten beobachten, daß EGF die Zellen dauerhaft depolarisiert, aber gleichzeitig die spannungsabhängigen Kaliumauswärtsströme um mehr als das Doppelte anstiegen. Welche Rolle dieser Befund für die EGF induzierte Proliferation von Gliomzellen spielt, ist noch unklar.