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POST-TRANSCRIPTIONAL REGULATION OF EXPRESSION OF THE POTASSIUM CHANNEL, Kv1.1

by

Margaret Louise Allen

A dissertation submitted in partial fulfillment
of the requirements for the degree of

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Chairperson of Supervisory Committee

Program Authorized to Offer Degree Department of Pharmacology

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Doctoral Dissertation

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University of Washington

Abstract

POST-TRANSCRIPTIONAL REGULATION OF EXPRESSION OF THE POTASSIUM CHANNEL, Kv1.1

by Margaret Louise Allen

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Potassium channels (K channels) are important in setting resting membrane potentials, shaping action potentials, modulation of hormone and neurotransmitter release and regulation of neuronal firing patterns (Hille, 1992; Jan and Jan, 1997). K channels are pivotal to many physiological functions and in fact a number of human diseases have been shown to be caused by K channel mutations (Biervert et al., 1998; Browne et al., Charlier et al., 1998; 1994; Singh et al., 1998; Zerr et al., 1998,). Since K channels are critical to normal physiology, it is important to understand regulation of expression of these genes. We report here identification of the transcriptional start sites and basal proximal promoter region for the mouse K channel, mKv1.1. Sequencing analysis reveals high sequence conservation of the

proximal promoter and 5'UTR regions of the mouse and human Kv1.1 genes. There is an intronic sequence in the 5'UTR of Kv1.1 that is present in 50% of cDNA clones (Tempel et al., 1988). We demonstrate that this intron is spliced out of mature, translated Kv1.1 RNA. In addition to the transcriptional elements, we demonstrate that the endogenously expressed Kv1.1 transcript in the C6 glioma cell line is rapidly destabilized with elevation of intracellular cAMP without a significant change in the transcription rate. This cAMP induced decrease in the steady state Kv1.1 RNA level is followed by a decrease in Kv1.1 protein and a decrease in a delayed-rectifier type K^+ current measured by whole-cell patch clamp electrophysiology. We demonstrate that Kv1.1 is important in maintaining the resting membrane potential in C6 glioma. Finally, we demonstrate that proliferation of C6 glioma is inhibited by conditions which destabilize the Kv1.1 transcript. This level of inhibition of proliferation is the same as that obtained when C6 glioma are cultured in the presence of the K channel blockers, 4AP and TEA. In conclusion, we have demonstrated that regulation of expression of the K channel, Kv1.1, occurs at the level of transcript stability and is correlated with elevation of intracellular cAMP concentrations. We speculate that this mechanism of regulation allows for rapid and dynamic control of expression of Kv1.1.

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LIST OF ABBREVIATIONS

4AP	4-aminopyridine
8-Br-cAMP	8 -Bromo- adenosine-3′,5′-cyclic Monophosphate
8-Cl-cAMP	8-Chloro- adenosine-3′,5′- cyclic Monophosphate
8-cpt-cAMP	8-(4-chlorophenylthio)-adenosine-3′,5′-cyclic Monophosphate
db-cAMP	N^6,$O^{2'}$-dibutyryl- adenosine-3′,5′-cyclic Monophosphate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAT	Chloramphenicol acetyltransferase
CHX	Cycloheximide
CNS	Central nervous system
CRE	cAMP response element
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTX-I	Dendrotoxin-I

EDTA	Ethylene-diamine-tetra-acetic acid
EF-1α	Elongation factor-1alpha
EGTA	Ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetracetic acid
GRE	Glucocorticoid response element
HEPES	(N-[2-Hydroxyethyl]piperacine-N′-[2-ethanesulfonic acid])
KCl	Potassium chloride
KOH	Potassium hydroxide
KRE	Kv1.5 repressor element
LDH	Lactate dehydrogenase
IBMX	3-isobutyl-methylxanthine
Iso	(-)-Isoproterenol
MAP kinase	Mitogen activated protein
NaCl	Sodium chloride
PDE	Phosphodiesterase
PKA	cAMP dependent protein kinase

PKG	cGMP dependent protein kinase
PMA	Phorbol 12-myristate 13-acetate
PNS	Peripheral nervous system
PKI	Inhibitor, cAMP dependent protein kinase
RPA	Ribonuclease protection assay
RMP	Resting membrane potential
SDS	Sodium doedecal-sulphate
TCA	Trichloroacetic acid
TEA	Tetraethylammoium
TES	N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid
TNF-α	Tumor necrosis factor-alpha
uORF	Upstream open reading frame
UTR	Untranslated region

Acknowledgments

The author wishes to thank the following individuals: Duk-Su Koh who was an invaluable collaborator. R. Jungmann for the LDH probe construct; B. Hille for supporting the electrophysiology studies; W. Schiemann for assistance with the PKA activity assay; W. K. Sonnenburg for the PDE profile, G. Mize with assistance with polysome gradients, F. Cartwright for assistance with photography, M. Coltrera for guidance with the cell proliferation assays, R. Veith for technical support. M. L. A. was supported by NIH training grant number GM07108. D.-S. K. was supported by NIH grant NS08174 (to B. Hille). This work was supported in part by NIH grants NS27206 and DC02739 (to B. Tempel).

DEDICATION

The author wishes to dedicate this dissertation to her husband W.K.S. and to give special thanks to her parents.

INTRODUCTION

Potassium channels (K channels) are important in various physiological functions such as maintaining resting membrane potential, cell volume regulation, shaping action potentials, modulation of hormone and neurotransmitter release and regulation of neuronal firing patterns (Hille, 1992; Jan and Jan, 1997). K channels have been identified in virtually all organisms including plants, bacteria, yeast and higher eukaryotes (Armstrong, 1998; Hille, 1992; Jan and Jan, 1997; MacKinnon et al., 1998). There are two major classes of K channels based on the 'rectification' of the channel - these are the inward rectifiers, Kir, and the Shaker-super family of K channels. Within each classification, families of channels are defined by the mechanism(s) regulating gating, such as stretch activation, ligand binding, nucleotide binding, Ca^{++} ions or changes in membrane potential (voltage gated) (for comprehensive reviews see: Hille, 1992; Jan and Jan, 1997; Nichols and Lopatin, 1997).

The first potassium channel was cloned from the *Drosophila* Shaker locus and was termed the Shaker K channel (Kamb et al., 1987; Papazian et al., 1987; Pongs et al., 1988; Schwarz et al., 1988; Tempel et al., 1987). Subsequent *Drosophila* K channel genes were cloned and called Shab, Shaw and Shal. Tempel et al. (1988) and Baumann et al. (1988) cloned the first mammalian

homologues of Shaker, MBK1 from mouse and RCK1 from rat, respectively. The K channel gene, *Kcna1*, has been mapped to mouse chromosome 6 (Street and Tempel, 1997) and the proteins and clones from this locus are labeled Kv1.1 (previously MBK1 and RCK1). In humans the Kv1.1 gene locus is called *KCNA1*. In the intervening years there has been an ever-expanding number of K channels cloned. The nomenclature has been standardized so that channels are designated Kv1.x (Shaker), Kv2.x (Shab), Kv3.x (Shaw) and Kv4.x (Shal) where the 'K' refers to potassium channel, 'v' indicates that it is voltage gated, and 'x' refers to the number of cloned sub-family member (Chandy and Gutman, 1993).

Complexity of Kv channel families

The topology of Kv channel alpha subunits consists of six trans-membrane domains, labeled S1 - S6, a pore domain between S5 and S6 which partially penetrates the membrane, and the amino and carboxy-termni which are both intracellular. The voltage sensor resides primarily in the S4 domain. Using immunoprecipitations with subunit specific antibodies it has been demonstrated that homo- and heteromultimers exist in vivo (Shamotienko et al., 1997; Sheng et al., 1993; Wang et al., 1993). Electrophysiology studies in expression systems and immunoprecipitation experiments indicate that there

is a constraint that heteromultimers form only between members of the same family, i.e. Kv1.1 + Kv1.3 but not Kv1.1 + Kv3.1 (Covarrubias et al., 1991; Hopkins et al., 1994; Po et al., 1993; Sheng et al., 1993). Furthermore, it has been demonstrated that heteromultimers have modified electrophysiological and pharmacological properties when compared to the relevant homomultimers (Christie et al., 1990; Hopkins et al., 1994; Isacoff et al., 1990; Po et al., 1993; Ruppersberg et al., 1990; and reviewed in Chandy and Gutman, 1993). Taking advantage of these changes in electrophysiological properties and the ability to create tandem alpha subunits with the differing K channel cDNA clones it was determined that four alpha subunits formed a functional channel (Heginbotham and MacKinnon, 1992; MacKinnon, 1991).

Recently, beta subunits (designated Kvβ1, Kvβ2 and Kvβ3, with Kvβ1 having 3 isoforms: Kvβ1.1, Kvβ1.2 and Kvβ1.3) have been cloned and co-expressed with the alpha subunits (Accili et al., 1997; Majumder et al., 1995; Morales et al., 1995; Rettig et al., 1994; Scott et al., 1994 and reviewed in Isom et al., 1994 and Jan and Jan, 1997). These beta subunits have been demonstrated to alter the gating kinetics (affecting both inactivation and activation parameters) of the K channel alpha subunits with which they are associated (Heinemann et al., 1994; Heinemann et al., 1996; Jing et al., 1997; Morales et

al., 1995; Rettig et al., 1994 and reviewed in Jan and Jan, 1997). As with alpha subunit co-assembly constraints, the beta subunits do not ubiquitously interact with all alpha subunits (Morales et al., 1995; Rhodes et al., 1995; Rhodes et al., 1997). An additional role postulated for the beta subunit is that it increases stability and promotes the surface expression of alpha subunits (Accili et al., 1997; Morales et al., 1995; Shi et al., 1996). Further, in remyelinating axons Rasband et al. (1998) postulate that beta subunits may direct the redistribution and localization of the alpha subunits. It has been postulated that beneficial physiological effects of the large number of various subunits allows for cells to modulate and fine-tune membrane characteristics by altering the K channel subunit composition.

Increased diversity in channel expression is also attained through alternative splicing. This is most apparent for the *Drosophila* Shaker gene in which at least 24 separate gene products have been identified and arise from extensive alternative splicing of the 5′ and 3′ ends of the coding sequence (Kamb et al., 1988). Alternative splice variants of Kv3.1 were shown to occur in an developmentally regulated manner in rat brain (Perney et al., 1992). More recently this group has shown that the switch in expression of the splice variants is regulated by differing signaling pathways. It was shown in these

studies that the increase in the transcript of one splice variant was sensitive to PKC inhibition in P8 animals but not in adult brain. Expression of the second splice variant was unaffected by PKC inhibitors at either age (Liu and Kaczmarek, 1998). A recent study demonstrated that alternative splicing of the Slo K channels (calcium and voltage activated) is regulated by stress hormones (Xie and McCobb, 1998). It was determined that adrenocorticotropic hormone (but not corticosteroids) increased the percentage of transcripts containing 'STREX' exon sequence. The electrophysiological properties of the STREX containing channels are that they activate more rapidly, deactivate more slowly and the voltage dependence of activation is shifted 20mV more negative. It is postulated that the STREX variant is expressed in the epinephrine and norepinephrine-secreting chromaffin cells which have been shown to exhibit repetitive firing. These differences in gating kinetics may enhance repetitive firing thus facilitating secretion of the stress hormones (Xie and McCobb, 1998). For the mammalian Shaker family of Kv channels Kv1.5 and Kv1.7 are reported to have an intron within the coding region (Attali et al., 1993; Chandy and Gutman, 1993). The intron described by Attali et al. (1993) for Kv1.5 results in a truncated transcript found in cardiac tissue and arises from an unusual,

putative splicing within the coding region. In addition to these examples of introns within the ORF, Kv1.1 and Kv1.4 both have introns within the 5'UTR regions (Tempel et al., 1988; Wymore et al., 1996). Further diversity of Kv1.4 occurs through differential use of poly-A^+ addition sites in the 3'UTR. Alternative splicing has been reported for the mammalian Shab family of channels (Luneau et al., 1991). For the beta subunits, the three Kvβ1 isoforms arise from alternative splicing of a single gene (England et al., 1995; McCormack et al., 1995). Thus, alternative splicing contributes to the diversity of functional K channel subunits.

Another post-transcriptional mechanism for generating K channel diversity is RNA editing. Patton et al. (1997) have demonstrated that this occurs in squid giving rise to diverse sqKv2 K channels (Patton et al., 1997). For this gene there are 17 positions with invariant adenosine residues within the genomic sequence (spanning S4 to S6 of the ORF) which are edited to guanosine in the cDNA sequence. The editing results in 28 unique transcripts with varying combinations of these residues 'edited'. At least two of the edited sequences give rise to K channels with altered deactivation and inactivation kinetics. Interestingly, this mechanism of RNA editing was first described for another ion channel, that of the GluR-B subunit of the

ionotropic glutamate receptor (Sommer et al., 1991). Thus, RNA editing extends the vast array of mechanisms that occurs to give rise to the diversity of K channel function.

Posttranslational regulation of Kv channel function

In addition to the subunit assembly, there are post-translational modifications which add sugar residues to the extracellular domains of the channels. Early work determined that alpha subunits from brain extracts contained both sialic acid residues and N-linked glycans (Rehm, 1989; Rehm et al., 1989; Scott et al., 1990). Consensus sites for N-linked glycosylation have been identified on the extracellular loops between S1 & S2 of Shaker K channels (Chandy and Gutman, 1993; Santacruz et al., 1994). Several K channels also have N-linked glycosylation consensus sites in the carboxy terminus region of the protein but these are unlikely to be modified as they are located intracellularly (Chandy and Gutman, 1993). Using insect cell culture and *Xenopus* oocyte expression systems Santacruz-Toloza et al. (1994) have demonstrated that glycosylation (using techniques of pharmacological inhibition of N-glycosylation and mutagenesis of the putative N-linked glycosylation consensus site) is not necessary for assembly or membrane targeting of functional Shaker potassium channels nor were any gating

kinetics altered. Using eukaryotic expression systems and very similar experimental approaches two groups, Deal et al. (1994) studying Kv1.1 and Spencer et al. (1997) studying Kv1.3, both concluded that N-linked glycosylation was not necessary for subunit assembly, expression or function. In contrast to these expression studies, Zona et al. (1990) determined that when N-linked glycosylation was pharmacologically inhibited by culturing primary cultures of rat neocortical neurons in tunicamycin that A-type K^+ currents were completely abolished and delayed rectifier K^+ currents were greatly reduced in early cultures (undifferentiated) but neither current type was reduced in older cultures (differentiated). These results led the authors to speculate that glycosylation was critically important in early development for proper channel expression and was less important in mature neurons. Although the above studies have demonstrated a relative lack of effect on electrophysiological parameters by N-linked glycosylation, a recent paper by Thornhill et al. (1996) showed that for Kv1.1 there was a positive shift in the activation voltage and slowed activation kinetics for channels lacking or with greatly reduced amounts of sialic acid modifications. It was postulated that the sialic acid residues contribute to the local electric field of the voltage sensor region of the channel and that the loss of these residues leads to the

measured shifts in gating kinetics. Similar results on gating kinetics of a delayed rectifier K^+ current were obtained with chemical modification of extracellular surface of the squid axon (Caputo et al., 1994). Since posttranslational modifications may vary from cell to cell, this type of modification, in conjunction with the subunit assembly, would allow for more fine tuning of electrical excitability with a limited number of gene products.

In addition to the inherent electrophysiological properties conferred by subunit combinations and glycosylation, K channel gating kinetics are modified on an acute time scale by phosphorylation. There are consensus PKC, PKA and tyrosine phosphorylation sites in the N- and C-terminal intracellular domains of the channel subunits. The effect of the differing phosphorylation or dephosphorylation states on kinetics varies by channel and kinase (for review see Jonas and Kaczmarek, 1996; Lewis and Cahalan, 1995; Siegelbaum, 1994). For example, for the lymphocyte K channel, Kv1.3, there are mixed reports of how phosphorylation/dephosphorylation affects gating kinetics. It seems that the effect of phosphorylation state on the channel depends on the system studied. Sloiven & Nelson (1990) and Cai & Douglass (1993) report that PKA decreases Kv1.3 peak current amplitude in T-

lymphocytes whereas, Wang et al. (1997) report that PKA increases the peak current amplitude of the K^+ current of myeloblastic ML-1 cells. Similar opposing effects of activation of PKC on Kv1.3 gating kinetics have been measured where Cai & Douglass (1993) report a decrease and Chung & Schlichter (1997) report an increase in Kv1.3 activation in T-lymphocytes. Since there may be more than one consensus phosphorylation site on a K channel subunit, it is likely that multiple phosphorylation/ dephosphorylation states can occur. Wilson & Kaczmarek (1993) have proposed that for K^+ currents in *Aplysia* bag cell neurons that the combined effects of kinases and phosphatases affect the gating state of the K channels. Thus, the electrophysiological kinetics of K channels can be affected by phosphorylation/dephosphorylation and that regulation of these interactions is complex and dependent on the cell-type or system used for study.

Transcriptional regulation of Kv channel expression

Additional mechanisms for achieving diversity of K channel expression is for the cell to regulate transcription of the genes encoding the proteins. The promoter regions of Kv1.3, Kv1.4, Kv1.5 and Kv3.1 have been cloned and some similarities have been identified. All of these Kv gene promoter regions are highly GC rich, lack canonical TATAAA and

GGCAATCT motifs and contain enhancer and/or repressor elements (Gan et al., 1996; Mori et al., 1995; Mori et al., 1993; Simon et al., 1997; Wymore et al., 1996). For Kv1.3 a minimal promoter has been identified in the 1.6kb of genomic sequence immediately upstream of the transcriptional start site. Deletion analysis of the promoter region cloned into a luciferase expression vector demonstrated that an enhancer region was contained in a 0.8kb span of the promoter. Primary sequence analysis of this 0.8kb region reveals that there are consensus elements for the transcription factors TFIID (3 sites), Sp1 (2 sites), and GGGCGG (1 site) (Simon et al., 1997). Which specific transcription factors actually regulate transcription has not yet been reported.

Wymore et al. (1996) have cloned the promoter for Kv1.4 and determined that the basal promoter region was contained within a 2.1kb region. Deletion analysis defined a minimal region of 464bp required for basal expression of reporter gene extracts. Primary sequence analysis reveals that the basal promoter region contains consensus sequence elements for Sp1 (3 sites), E-boxes ('multiple' sites), fibroblast enhancer and AP2 (1 site for each). As for the Kv1.3 promoter, no further transcriptional analysis of Kv1.4 has been reported.

Mori et al. (1993) have genomic clones of Kv1.5 and have analyzed 2.3kb of promoter and ~0.8kb of 5'UTR by cloning these into expression vectors. They have determined that the promoter functions in a tissue specific manner - with significant expression only in mytubes or C2C12 cells but not in HeLa or myoblasts cells. Furthermore, they identified an CRE element in the 5'UTR region of the genomic unit and found that this conferred cAMP included increases in reporter gene activity in transfection experiments with reporter gene constructs. Mutagenesis of the CRE element abolished the cAMP induced increase in reporter gene activity. Electromobility gel shift assays were used to demonstrate binding of CREB and CREM - two members of the CRE binding family of transcription factors. Primary sequence analysis also demonstrated that there was an consensus site for GRE (glucocorticoid response element) 5' of the CRE, both of which were initially mapped as residing in the 5'UTR of the transcript. In a later paper this group (Mori et al. 1995) redefined the transcriptional start sites which place the CRE and GRE upstream of these 'new' transcriptional start sites. These authors also identified a repressor element, named KRE (Kv1.5 repressor element), that is postulated to be responsible for the cell type specific expression of this gene. Again using electromobility shift assays with a KRE

specific probe, they demonstrated that DNA-protein interactions occurred only with nuclear extracts from cells which express Kv1.5 (GH3) but not from non-expressing cells (CHO & COS-7). It is likely that these various promoter elements underlie the tissue specific changes in Kv1.5 transcript levels with differing pharmacological treatments. For example, with elevation of cAMP there is a 6-fold increase in Kv1.5 mRNA in cells of cardiac origin but a 6-fold decrease in Kv1.5 mRNA in cells of pituitary origin (GH3 cells) (Mori et al., 1993). Furthermore, cAMP and PKA activity was required for maintained basal expression of Kv1.5 in GH3 cells (Takimoto et al., 1995). Dexamethasone was shown to rapidly increase transcription of Kv1.5 in GH3 cells (Levitan et al., 1991; Takimoto et al., 1993), increased ventricular expression but had no effect on atrial expression of Kv1.5 (Levitan et al., 1996; Takimoto and Levitan, 1994). This series of studies clearly demonstrates that the tissue specific dynamic regulation of expression of Kv1.5 can occur at the level of transcription.

Gan et al. (1996) have cloned and analyzed the promoter for Kv3.1. Sequence analysis of 1.5kb of the proximal promoter regions determined that it contained consensus sequence elements for Sp1 (5 sites, clustered close together), Ap-1 and CRE (1 site each) and "other" transcription factor binding

sites. Reporter gene constructs were made by inserting 5.3kb of proximal promoter sequence upstream of the reporter gene, CAT (chloramphenicol acetyltransferase). Transfection data revealed that expression was 50-fold higher in PC12 cells than in NIH3T3 cells. Deletion analysis of the reporter constructs demonstrated that the four most proximal Sp1 sites were required for basal promoter activity but that neither the Ap-1 nor the CRE were required. Not surprisingly, the CRE element was shown to be required for cAMP stimulated increases in reporter gene activity. Finally, electromobility shift assays were used to show that CREB bound to probes derived from the CRE element region of the promoter.

Translational regulation of Kv channel expression

Very few reports have been published where regulation of K channel expression is regulated at the translational level. This is somewhat surprising since at least for the Shaker family of channels there are three members, Kv1.1, Kv1.2, and Kv1.3 which each have intronless ORFs of approximately 1.5kb in length and yet the mature transcript sizes for these range from 8 to 9.5kb. Thus, there is a very large amount of UTR sequence in the transcripts of these genes which may allow for regulation of expression by regulating translational efficiency, transcript stability or subcellular trafficking. Kv1.4

has a very long 5'UTR with 8 upstream AUGs (Wymore et al., 1994) and this UTR region is highly conserved between species (hKv1.4 and mKv1.4 5'UTR is 74% conserved). In addition to the long, highly conserved 5'UTR of Kv1.4, Wymore et al. (1996) have demonstrated that the two transcripts arising from this gene diverge in the 3'UTR. The difference is likely due to alternative poly-A^+ addition site usage. The apparent functional consequence is that the longer transcript species contains more AUUUA motifs. These motifs have been associated with transcript instability. Therefore, it is consistent that the data show a lower current density and protein expression of constructs containing the longer 3'UTR relative to the shorter 3'UTR sequences (Wymore et al., 1996).

Diseases caused by K channel mutations

Although there is a great diversity of K channel subunits and the expression of many of these overlap, mutations or deletion of one type of K channel subunit can have deleterious physiological consequences. In an experimental model system the neuronal expression patterns of the mRNAs for Kv1.2 and Kv4.2 but not Kv1.1 and Kv1.4 were shown to be altered by induced seizures (Tsaur et al., 1992) or by administration of electroconvulsive shock (Pei et al., 1997). Specifically, following either treatment paradigm the

in situ hybridization signal for Kv1.2 and Kv4.2 were reduced transiently in the hippocampus, but no reduction in Kv1.1 or Kv1.4 were detected. Hypoxia has also been demonstrated to alter expression patterns of voltage gated K channels, but this seems to be cell type specific in terms of the regulation. For example, Conforti & Millhorn (1997) used RT-PCR to show an increase in Kv1.2 mRNA in PC12 cells cultured under conditions of hypoxia, they detected no changes in Kv1.3, Kv2.1, Kv3.1 or Kv3.2. In contrast to these results, Wang et al. (1997) demonstrated that prolonged hypoxia induced decreases in both mRNA and protein levels of Kv1.2 and Kv1.5 in primary cultured rat pulmonary arterial smooth muscle cells. In yet another system - a renovascular hypertensive rat model - ventricular expression of Kv4.2 and Kv4.3 were decreased but no changes were detected for Kv1.2, Kv1.4, Kv1.5, Kv2.1 or KVLQT1 in rats with induced hypertension (Takimoto et al., 1997). In none of these systems was the mechanism, transcriptional or posttranslational, of the changes in expression levels determined.

In the experiments mentioned above the physiology was manipulated (seizure, hypoxia) and changes in K channel expression was monitored. In another approach, specific K channel (Kv1.1) expression is targeted and the consequences on physiology and or behavior are monitored. The first of

these is a mouse knock-out of Kv1.1 where the phenotype of homozygote animals is primarily epilepsy that is often fatal. In addition, these Kv1.1 knock-out animals show changes in nerve conduction and hyperexcitability in hippocampal slice electrophysiological recordings (Smart et al. 1998). In another model system that is less severe than a knock-out, anti-sense oligodeoxyribonucleotides (aODN) directed to Kv1.1 were injected into the intracerebroventricles of rodents to reversibly and specifically decrease expression of Kv1.1 in the brains of the treated animals. As a result of these injections, the animals displayed increased feeding behavior (Ghelardini et al., 1997), an inhibition of the antinociception induced by μ-opoid agonists morphine and baclofen (Galeotti et al., 1997), and impaired associative memory in the paradigms of passive avoidance and spatial memory (Meiri et al., 1997). In the latter study electrophysiological measurements of hippocampal slices demonstrated a prolonged repolarization and no after-hyperpolarization in recordings of dentate granule cells. No alterations were seen with measurements designed to detect LTP. Since no data was presented to demonstrate that Kv1.1 expression was completely eliminated in the slices from which the recordings were made, it is difficult to conclude that there is a

cause/effect link between the aODN mediated reduction in Kv1.1 and the memory changes.

As would be predicted from these targeted K channel model systems, the underlying cause of several human diseases have recently been determined to be mutations in K channels. For example, point mutations in the coding region of the Kv1.1 channel have been demonstrated to give rise to the human inherited neurological condition, episodic ataxia/myokymia syndrome (Browne et al., 1994). When cDNA containing the various point mutations were expressed the resulting channels had diminished current amplitudes and altered gating kinetics (Zerr et al., 1998). In another example, point mutations in another K channel, KVLQT1, have been demonstrated to underlie the human electrocardiographic phenotype of long QT syndrome caused by ventricular arrhythmia. This is an autosomal dominant disease that is often fatal. In a final example, another autosomal dominant human disease, benign familial neonatal convulsions (BFNC) - a form of idopathic generalized epilepsy, was shown to link to two chromosomes 8q24 and 20q13. Recently, the BFNC linked with 20q13 was demonstrated to arise from frame shift mutations in a new K channel gene, KCNQ2 (Biervert et al., 1998; Singh et al., 1998). The form of BFNC linked to 8q24 was shown to be a point

mutation in another new K channel, KCNQ3 (Charlier et al., 1998). Interestingly, this point mutation in KCNQ3 is the same mutation that occurs in the related K channel gene, KVLQT1 (Charlier et al., 1998).

In summary, there are many means of regulation of expression and regulation of activity of K channels. Perturbations of expression, whether through mutations or removal of subunits can cause severe physiological disorders. Thus, it is important to understand these facets of regulation of K channel expression.

CHAPTER 1: MAPPING AND ANALYSIS OF THE 5′ REGION OF THE POTASSIUM CHANNEL Kv1.1

INTRODUCTION

Voltage-gated potassium channels (Kv channels) are important in maintaining resting membrane potential, shaping action potentials, modulation of hormone and neurotransmitter release and regulation of neuronal firing patterns (Hille, 1992; Jan and Jan, 1997). The first potassium channel was cloned from the *Drosophila* Shaker locus and was termed the Shaker K channel (Kamb et al., 1987; Papazian et al., 1987; Pongs et al., 1988; Schwarz et al., 1988; Tempel et al., 1987). Subsequent *Drosophila* K channel genes were cloned and called Shab, Shaw and Shal. Tempel et al. (1988) and Baumann et al. (1988) cloned the first mammalian homologues of Shaker, MBK1 from mouse and RCK1 from rat, respectively. The K channel gene, *Kcna1*, has been mapped to mouse chromosome 6 (Street and Tempel, 1997) and the proteins and clones from this locus are labeled Kv1.1 (previously MBK1 and RCK1). In humans the Kv1.1 gene locus is called *KCNA1*. In the intervening years there has been an ever-expanding number of K channels cloned. The nomenclature has been standardized so that channels are designated Kv1.x (Shaker), Kv2.x (Shab), Kv3.x (Shaw) and Kv4.x (Shal)

where the 'K' refers to potassium channel, 'v' indicates that it is voltage gated, and 'x' refers to the number of cloned sub-family member (Chandy and Gutman, 1993).

Cloning and expression has greatly facilitated the electrophysiological, biochemical (subunit interactions), pharmacological and mRNA (in situ hybridization) and protein (immunocytochemical) tissue distribution of for K channels. In many of these studies it was only necessary to use the cDNA encoding the ORF - excluding even the UTR regions. In contrast to the volumes of information learned from using the cDNAs, recent investigations have begun to map the K channel transcriptional start sites and conduct analysis of promoter regions. The promoter regions of Kv1.3, Kv1.4, Kv1.5 and Kv3.1 have been cloned and some similarities have been identified. All of these Kv gene promoter regions are highly GC rich, lack canonical TATAAA and GGCAATCT motifs and contain enhancer and/or repressor elements (Gan et al., 1996; Mori et al., 1995; Mori et al., 1993; Simon et al., 1997; Wymore et al., 1996). The minimal basal promoter for Kv1.3 is contained within 800bp (Simon et al., 1997) and for Kv1.4 is contained within 464bp (Wymore et al., 1996) of the respective promoter regions. The promoter for Kv1.5 has been the most extensively studied and shown to contain the following transcription factor consensus sites: CRE (cAMP response element), GRE (glucocorticoid response element) and a newly identified repressor KRE

(Kv1.5 repressor element) (Mori et al., 1995; Mori et al., 1993). These different elements have been postulated to be responsible for the differing tissue specific pattern of expression of Kv1.5 (Levitan et al., 1991; Levitan et al., 1996; Mori et al., 1995; Mori et al., 1993; Takimoto et al., 1993; Takimoto et al., 1995; Takimoto and Levitan, 1994). The promoter for Kv3.1 has also been analyzed and among several transcription factor consensus sites a CRE was demonstrated to be necessary for cAMP induced expression of reporter activity in transfection experiments with reporter constructs (Gan et al., 1996).

The transcript for Kv1.1 is ~8kb in length with an intronless ORF of 1.5kb. A putative intron of 368bp within the 5'UTR was reported in 50% of the original cDNA clones; for the longer clones the 5'UTR was 1.3kb (Tempel et al., 1988). Baumann et al. (1988) cloned the rat homologue and this cDNA clone, in addition to the ORF, contained 2.1kb of 3'UTR sequence. Thus, there is ~ 3kb of sequence that is unaccounted for in this ~8kb transcript. We set out to 1) define the transcriptional start site(s) for Kv1.1; 2) to define and analyze the basal promoter region of Kv1.1; and 3) to sequence the 5' region of hKv1.1 for comparison to mKv1.1 sequence.

METHODS

Reagents

Cell culture media was purchased from GIBCO/BRL. Serum for cell culture was purchased from HyClone. Oligo-dT cellulose was purchased from

Collaborative Research. ^{32}P-radionucleotides were purchased from NEN. Molecular biology reagents were purchased from Boehringer Mannheim Biochemicals. Sequencing was done using ABI fluorescent dye-terminator sequencing kits. Eukaryotic expression vectors were purchased from Promega. Other standard biochemical reagents were purchased from Sigma.

RPA Analysis:

RNA was extracted from the cells using guanidininum as described (Chomczynski and Sacchi, 1987). For RNase Protection Assay (RPA) varying amounts of either of total or Poly-A$^+$ selected RNA was incubated ^{32}P-radiolabeled riboprobes (specifics detailed in figure legends). The RPA protocol is that of (Bordonara et al., 1994) except that riboprobes were synthesized with 0.4μM UTP in the reaction mixture. In the hybridization, 5 x 10^5 cpm of riboprobe was included per sample. The dried gels were exposed to a phosphorimager screen for quantitative analysis. The 5'Kv1.1 riboprobe was as previously described (Bosma et al., 1993). Riboprobe KP535 spans the KpnI to PstI site of the genomic clone of Kv1.1 and was subcloned into the KpnI and PstI sites of pBluescript vector. Riboprobe template for β-actin was purchased from Ambion.

Northern Analysis

Aliquots of 10μg of poly-A^+ RNA were run on a 1% agarose gel in 18% Formaldehyde/HEPES buffer. The gel was nicked by soaking gel in 50mM NaOH, 100mM NaCl for 30 minutes followed by two washes in water. The RNA was then transferred to Nytran membrane in 20XSSC. The blots were baked in a vacuum oven for 2 hours. The blots were blocked for 4 hours at 50°C with prehybridization solution composed of: 50% formamide, 6XSSC, 2X Denhardt's, 50mM sodium phosphate, pH 7.0, 0.3mg/ml denatured salmon sperm DNA. The labeled probes were boiled and then added directly (in ≤200μl) to the prehybridization solution and incubated O/N at 50°C. Blots were washed 15 min. each with: 2X SSC/1% SDS at RT (twice); 1X SSC/0.5% SDS at 60°C; 0.5X SSC/0.5% SDS at 60°C; 0.4X SSC/0.5% SDS at 60°C; 0.2X SSC/0.5% SDS at 65°C. For probe generation, the regions of the genomic clone (as indicated in the figures) were isolated and nick-translated using random hexanucleotide primers.

S1 Nuclease Assay

The S1 nuclease assay was performed according to the protocol in Current Protocols in Molecular Biology (Ausubel et al., 1991) with the following exception: the denaturing gel was 1.3% agarose and containing 30mM NaCl and 2mM EDTA. After gelling it was soaked for a minimum of 4 hours to O/N in alkaline running buffer, 30mM NaOH, 2mM EDTA. Gels

were run for 4 to 4.5 hours at ~65V, with gel box packed in ice. End-labeled probes were prepared as described in Current Protocols in Molecular Biology (Ausubel et al., 1991) from either single stranded DNA templates or from denatured double stranded template. Sequence of oligo 465: 5'-CGCTCGGTCCATTTCTGGGCGCTGC-3'; sequence of oligo 886: 5'-CCCTCTGAACACTACGGGAGCCTTGATTCG-3'.

Sequencing

Sequencing was done using ABI fluorescent dye-terminator sequencing kits. Reactions were run and processed by the Department of Pharmacology core facility.

Transfections

The KpnI to NotI sequence of the genomic clone was cloned into the KpnI and NotI sites of the pGL2-Basic and pGL2-Enhancer eukaryotic expression vectors from Promega. Two deletions within each were made by deleting the KpnI to PstI (5' half) or the PstI to NotI (3' half) in each vector (Basic and Enhancer). C6 Glioma were transfected using electroporation (BTX square wave electroporator, 4 x 10^6 cells + 25μg plasmid DNA/2mm gap cuvette, 0.8Kvolts, 50μsec pulse, 5 pulses). Cells were harvested ~20 hours after electroporation for Luciferase assay. Luciferase assay protocol was from Current Protocols in Molecular Biology (Ausubel et al., 1991). Luminescence was measured on a Berthold luminometer.

RESULTS

Transcriptional start site mapping

Analysis of an 8.5kb genomic clone that is likely to encompass the entire 5'UTR region upstream of the 5' end of mKv1.1 cDNA suggests that the transcriptional start site is between the PstI and NotI sites (Fig. 1). Sequencing of the HindIII to HindIII fragment of this genomic clone indicates that there may be a second, unidentified ORF 4kb upstream of the NotI site (see map in Fig. 1) (Hallows, Allen & Tempel, unpublished observation). When Northern blots are probed with this HindIII/HindIII fragment a transcript is detected that is clearly not Kv1.1 (data not shown) which further confirms that another ORF is located in this region. Northern blots with probes generated from adjacent regions of the genomic clone (see Fig. 1 for details) has demonstrated that only the probe derived from PstI to NotI was able to detect a transcript of the same size as the ORF probe. Also, if comparing the signal intensity, the signal obtained with this probe is very weak relative to the ORF probe, indicating that it may only be a very small region within the PstI to NotI region that hybridizes to the Northern. This result was obtained with two separate sets of Northerns with differing batches of poly-A^+ RNA.

RPA analysis using a riboprobe that encompasses the KpnI to PstI region of the genomic clone (probe KP535, see Fig. 1 and Fig. 2) was unable to detect any protected fragments (Fig. 2). This result is consistent with the

previous Northern analysis. Several attempts were made to map the 5′ transcriptional start site(s) using RPA analysis of the region from PstI to NotI (see Fig. 1). We were unable to synthesize full-length riboprobes which we speculate is due to the very high GC content of the region near the NotI site. A few attempts were made to do primer extension and 5′ RACE, again neither protocol worked. We do not know if the difficulty with these latter two techniques was the high GC content of the sequence and/or the fact that the transcript for Kv1.1 is not an abundant message. Therefore, we used S1 nuclease assays to map the transcriptional start sites. Using an end-labeled probe generated with oligo 886 (Fig. 4) and 5μg poly-A^+ RNA/sample we protected a less-than full-length fragment with a 2 or 3 band cluster mapping to -1029 (Fig. 3A). A second end-labeled probe generated with oligo 465 (Fig. 4) and 50μg poly-A+ RNA/sample shows a cluster of start sites beginning at -1201 (Fig. 3B).

Basal promoter region of Kv1.1

Complete sequence analysis of a 1.2kb genomic fragment (-2126 (KpnI) to -1247 (NotI)) has revealed no TATAA or GGCAATCT transcriptional start consensus elements (Fig. 4). A cluster of Sp1 consensus sites is found 50 to 60bp upstream of the mapped transcriptional start site cluster beginning at -1201 and ~80bp upstream of the transcriptional start site cluster at -1029 (Fig.

4). This relationship of Sp1 site clusters to transcriptional start sites has been previously shown to occur for transcriptional initiation that occurs for TATAA- and CAAT-less promoters (Lu et al., 1994).

In addition to the Sp1 sites, sequence analysis identified a large number of consensus sites for other transcription factors. Of these, two that may be important (based on expression localization) are: NeuroD and Krox24 (also known as NGFI-A - nerve growth factor inducible gene A and EGR1 - early growth response 1 and zif/268). The consensus sites for these two transcription factors are indicated in Fig. 4. Notably lacking from this analysis is a CRE (cAMP response element).

To test this region for basal promoter activity, the KpnI to NotI fragment (Fig. 1 and Fig. 5) was cloned into Promega's Luciferase expression vectors, one without promoter or enhancer elements (basal) and the other lacking a promoter but with an enhancer element (enhancer). Convenient restriction sites (KpnI, PstI and NotI) were used to make 5′ and 3′ deletions (Fig. 5). These constructs were transfected into C6 glioma cells and extracts were assayed for reporter gene activity. This KpnI to NotI genomic region functioned as a basal promoter and deletion of the proximal region, PstI to NotI, reduced the level of reporter gene activity to that of the respective vector lacking a promoter (Fig. 5). Deletion of the distal region, KpnI to PstI, may remove an inhibitor element as there is an increase in reporter gene

activity compared to the construct with the 'full-length' KpnI to NotI promoter region (Fig. 5).

To investigate if cAMP influenced transcriptional activity, we treated transfected cells with 8-cpt-cAMP and looked to see if reporter gene activity would be either reduced or increased above untreated, transfected cells. As can be seen in Fig. 5 we detected no change with cAMP treatment.

Sequence and homology between hKv1.1 and mKv1.1 5'UTRs

The genomic sequence of hKv1.1 encompassing the 5'UTR and proximal promoter region was subcloned into M13 vectors for sequencing. Sequencing was done with the ABI fluorescent dye-terminator sequencing system. Sequence alignment (using the GAP subroutine of GCG University Wisconsin GCG program) comparing mKv1.1 and hKv1.1 is presented in Fig. 6. Analysis of the 5'UTRs revealed that there were multiple uORFs in this region. For mKv1.1, 6 of 8 uORFs, and for hKv1.1, 8 of 9 uORFs, the putative translated peptide contain 5 or more amino acids (Table 1 and 2). Of these, there are 5 uORFs for mKv1.1 and 6 uORFs for hKv1.1 that have good Kozak consensus for translation initiation (Kozak, 1987). For both mKv1.1 and hKv1.1, 4 of the uORFs have the translation start (AUG) within the intron and are thus not likely to be a feature of translational regulation in the mature transcript (see following chapter for discussion). The positions of the AUGs were close but not absolutely conserved between species. For two

uORFs the translated peptides are conserved in 10 of 17 and 11 of 20 positions between mouse and human sequence (Table 1 and Table 2). The alignment of these translated uORFs are shown here with '*' indicating conserved amino acids.

```
mKv1.1 (-1433)    MDRANLRPHAPCPTRSC
                  ****    * *** * *
hKv1.1 (1469)     MDRADPPPGAPCSTPSS


mKv1.1 (-1110)    MGVGVGRFLRSCSIKRSDDS
                  ****    * **** **
hKv1.1 (-1135)    MGVGKEHFRRSCSVKRLSN
```

In addition to the conservation in the primary sequence and the uORFs, there is likely to be conservation in secondary structure. In fact, a stem-loop structure is predicted for mouse, rat and human transcripts at positions -76 to -98 (mKv1.1 numbering) As can be seen in Fig. 7, of the 12bp stem of this predicted stem-loop structure there are 11 G-C base pairs. Since G-C base pairs are stronger than A-T base pairs, this predicts a very stable structure (Tinoco et al., 1973). In support of this, it was found in the initial cloning and analysis of this cDNA that to obtain expression in *Xenopus* oocytes, this region had to be removed from the transcript.

Finally, the overall 5'UTR sequence conservation between mKv1.1 and hKv1.1 is 78% with several stretches of sequence with conservation of 90%

(Fig. 8). Note that the conservation of the ORF is 96% and the conservation in the 3'UTR is 38% (comparing 840bp hKv1.1 3'UTR sequence with 2.1kb rKv1.1 3'UTR sequence).

DISCUSSION

Previous work in the Tempel lab has indicated that a delayed-rectifier type potassium channel, mKv1.1, is uniquely expressed in a subset of neurons in the mouse brain that support the accurate timing of action potentials. Recent work by H. Wang (1993, 1994) has shown that the mKv1.1 channel protein is localized at nodes of Ranvier in axons and in nerve terminals, both of these regions are critical for regulation of electrical activity. Furthermore, Wang et al. (1995) and Rasband et al. (1998) demonstrated that in dismyelinating conditions the expression pattern of Kv1.1 is altered. Additionally, Hallows and Tempel (1998) have determined that there is a complex pattern of Kv1.1 expression during development. Thus, because of the critical importance of Kv1.1 to correct neuronal firing patterns and (as will be shown in chapter 3) in maintaining resting membrane potential in glial cells, it is important to understand how expression of Kv1.1 is regulated.

The mapping of the transcriptional start sites for Kv1.1 and analysis of the primary sequence has demonstrated that transcription of Kv1.1 is through TATA-less, CCAAT-less, multi-start site initiation. The most frequent

transcription factor consensus binding sites were those for Sp1 which occurs in large clusters positioned upstream of each of the mapped transcription start sites. The positional proximity of the Sp1 site clusters relative to the transcriptional start sites is consistent with TATA-less/CCAAT-less promoters. Another transcription factor for which a consensus site was indicated is NeuroD. This may be interesting because this transcription factor was first described as being implicated in neurogenic differentiation (Lee et al., 1995). A consensus site for Krox24 was also found in the promoter region of Kv1.1. Krox24 is also known as EGR1 (early growth response 1), NGFI-A (nerve growth factor inducible gene) and zif/268 (Bravo, 1990). Krox24 is most abundantly expressed in brain (Lemaire et al., 1988; Sukhatme et al., 1988), has been implicated in G0/G1 transitions (the relevance of this will be discussed in chapter 5) (Bravo, 1990; Janssen et al., 1989), and there are two reports that Krox24 may act as a repressor (Ackerman et al., 1991; Christy and Nathans, 1989). This latter point is interesting considering the consensus site for Krox24 (-2120 to -2112) is in the distal (KpnI to PstI) proximal promoter region and when this is deleted from expression vectors there is an increase in reporter gene activity when the constructs are transfected in to glioma cells.

A feature of the Kv1.1 transcript is that the 5'UTR is very long, making it one of the only 5 - 10% of eukaryotic mRNAs so far identified with long (600 - 1200bp) 5'UTRs (Jackson et al. 1990). Also of interest is that within this

long 5'UTR there are 8 (mKv1.1) or 9 (hKv1.1) AUG translation initiation start codons. One of these codons is in frame with the authentic ORF and conforms to the Kozak consensus for ribosome initiation but, is unlikely to alter the authentic ORF as it resides within the intron. For both mKv1.1 and hKv1.1 there are 4 uORF start sites within the intron and are thus not likely to influence translational regulation of the Kv1.1 transcript. Two of the uORFs in hKv1.1 and mKv1.1 are conserved in approximate relative position (5' end of UTR region) and have 11 of 20 and 10 of 17 identical amino acids in the putative translated peptide sequence. It is unknown at this time if either of these uORFs have regulatory functions like the uORFs in the S-adenosylmethionine decarboxylase gene (Hill and Morris, 1993), gp 48 gene of cytomegalovirus (CMV) (Degnin et al., 1993) and arg-2 gene of Neurospora (Luo and Sachs, 1996) (also see review by Lovett and Rogers, 1996).

Preliminary sequence analysis revealed a high degree of sequence conservation in the 5'UTR regions but not in the 3'UTR regions. In this analysis the conservation of the sequence encoding the ORF between mouse, rat and human was ≥96%. The available 250bp of 5'UTR sequence of the rat, rKv1.1, is 96% identical to the mouse mKv1.1 5'UTR sequence. Similarly, when we compared the 1.2kb of 5'UTR sequence of the human transcript, hKv1.1, to that of the 1.2kb of mKv1.1 the overall conservation is 78% with several regions of ≥90% conservation. In contrast to the high conservation in

the 5'UTR region, there was only about 40% conservation in the 3'UTR sequence (~150bp mKv1.1, 1.4kb of rKv1.1 and 840bp of hKv1.1). This high conservation of 5'UTR sequence between species is unusual and is not seen, for example, with a neuronally expressed phosphodiesterase in which the bovine and human homologs show only 48% identity in the 5'UTRs. Likewise, this is not a general feature of ion channels as there is no identity or homology in the 5'UTR between sodium or calcium channel α-subunits as determined by analysis of sequences entered into GenBank. Among K channels, Kv1.2 and Kv1.4 have relatively high sequence conservation (75 to 80%) in the 5'UTR between species.

In conclusion, we have defined the proximal promoter for Kv1.1 which is sufficient to drive expression of luciferase in a eukaryotic expression system. The transcriptional start sites are consistent with TATA- and CCAAT-less multi-start site initiation. The transcript for Kv1.1 has a 5'UTR that is ~1kb in length, is highly GC rich, has putative conserved stem-loop structures, contains numerous uORFs and is highly conserved between species - features which may suggest that it is under translational regulation.

Fig. 1. Genomic structure of mKv1.1 gene and location of putative intron location within the 5'UTR of the transcript. Northern analysis to determine transcriptional start of Kv1.1. Poly-A^+ RNA from mouse brain (B) and liver (L) were run in sets of adjacent lanes. Probe X->K represents the XbaI to KpnI region of genomic clone, probe K->P represents the KpnI to PstI region of the genomic clone, probe P->N represents the PstI to NotI region of the genomic clone, and ORF refers to a probe generated from the ORF of Kv1.1. Blots were reprobed with EF-1α as control.

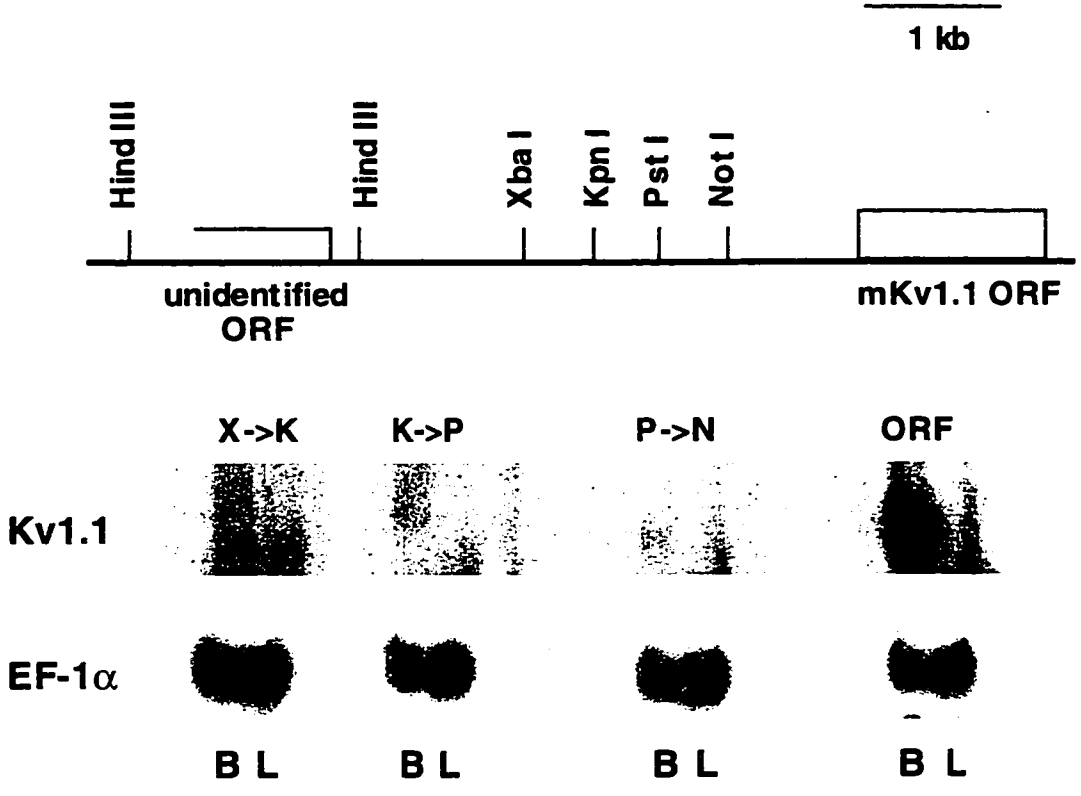

1 kb
Hind III
Hind III
Xba I
Kpn I
Pst I
Not I
unidentified ORF
mKv1.1 ORF
X->K
K->P
P->N
ORF
Kv1.1
EF-1α
B L
B L
B L
B L

Fig. 2. RPA analysis to determine transcriptional start of Kv1.1. Riboprobes were made to the regions indicated on the map at the bottom of the figure. Lanes 1 to 3 are probe 5′ Kv1.1 and lanes 4 to 6 are probe KP535. Lanes 1 and 4 are the respective full-length probes, lanes 2 and 5 are tRNA, and lanes 3 and 6 are poly-A^+ mouse brain RNA.

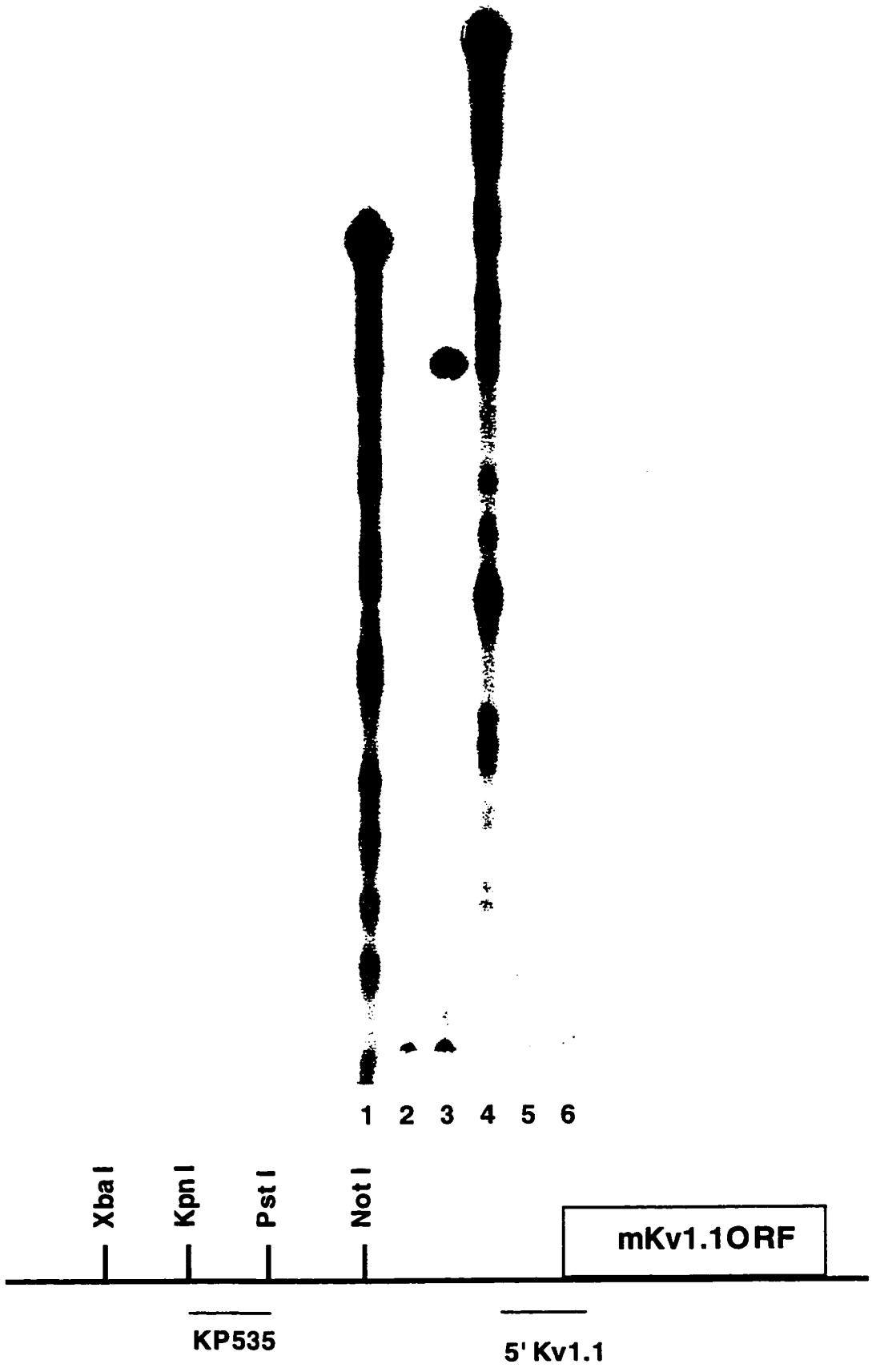

1 2 3 4 5 6
Xba I
Kpn I
Pst I
Not I
mKv1.1ORF
KP535
5' Kv1.1

Fig. 3. S1 Nuclease Mapping of Kv1.1 transcriptional start sites. **A.** Probe 886, mapping a cluster of 2 to 3 sites at -1029 and also showing full-length protection. **B.** Probe 465 showing cluster of start sites at -1201. Probe lane marked P, tRNA lane marked t, mouse brain poly-A^+ RNA marked B, mouse liver poly-A^+ RNA marked L. Panel A sequencing ladder run adjacent to sample lanes. Panel B radiolabeled 1 kb DNA ladder run adjacent to sample lanes.

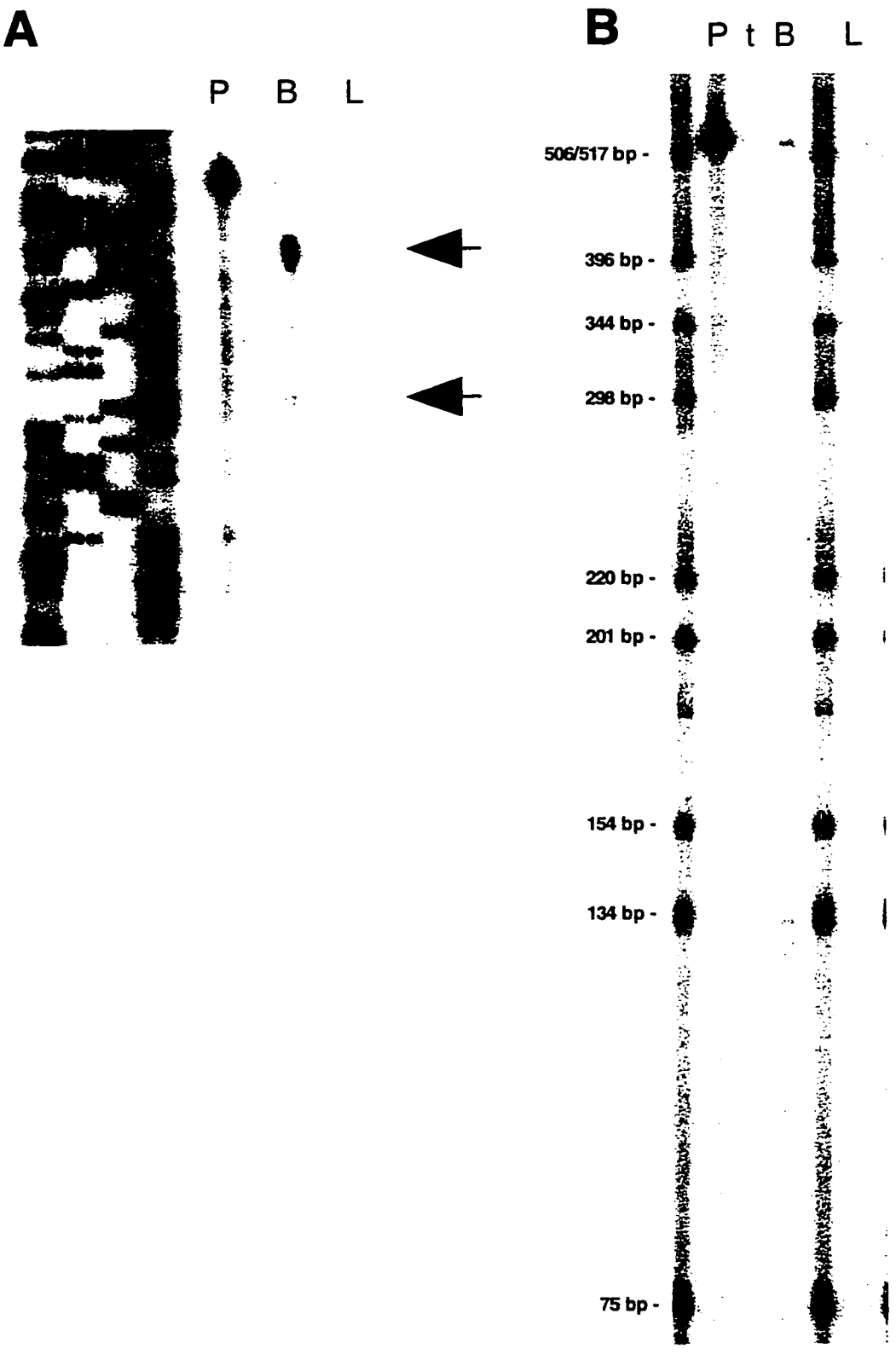
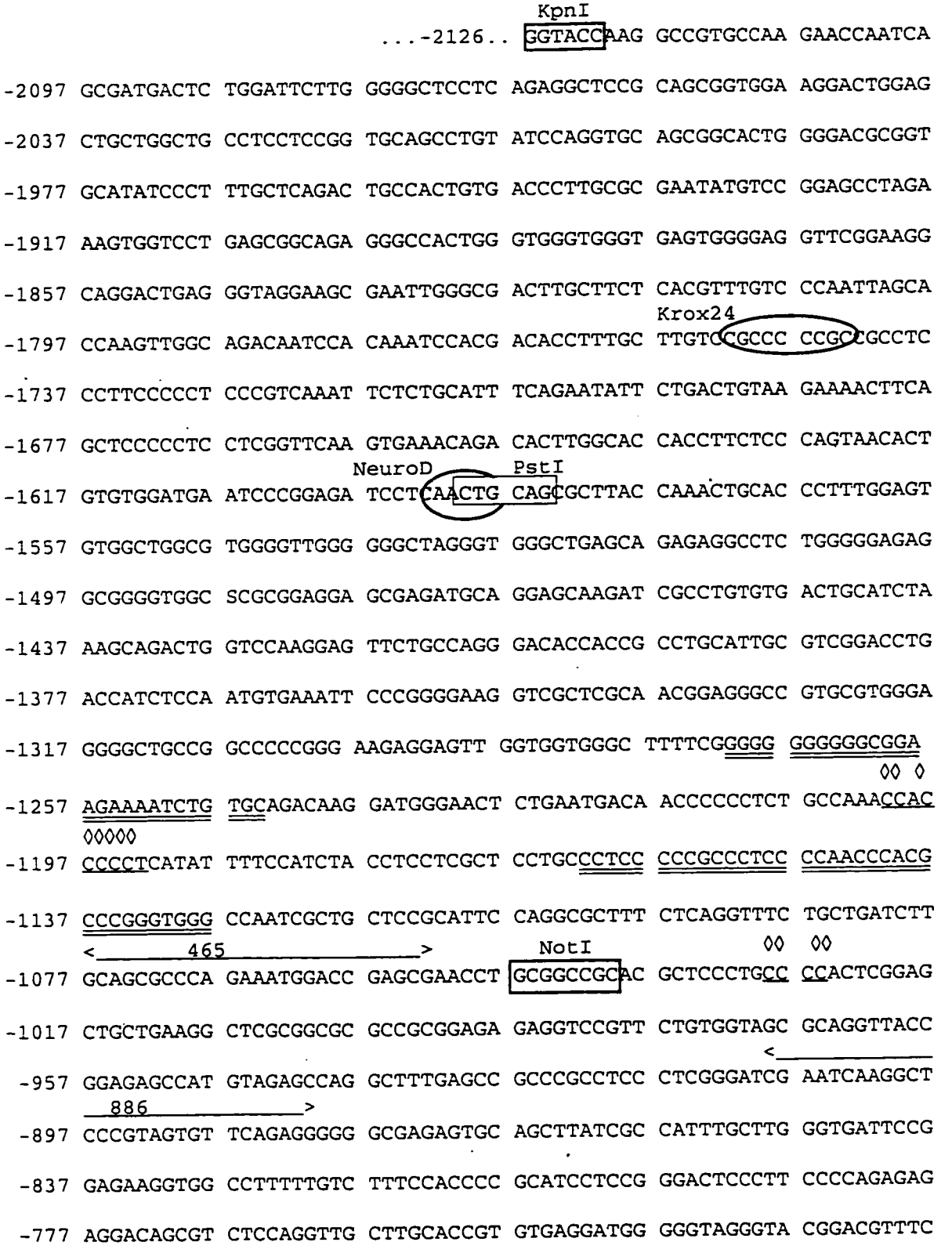

A
P
B
L
B
P t B L
506/517 bp -
396 bp -
344 bp -
298 bp -
220 bp -
201 bp -
154 bp -
134 bp -
75 bp -

Fig. 4 Sequence of mKv1.1 proximal promoter and 5'UTR. Positions of restriction sites are indicated with the name above lightly shaded boxes. Position of oligos used for generation of end-labeled probes for S1 nuclease analysis indicated by <___> with the number of the oligo between the <>. Transcriptional start sites indicated by ◊. Position of Sp1 clusters indicated by double underline. Transcription factor consensus sites for Krox24 and NeuroD are indicated by shaded ellipses. Note: intron sequence is not represented.


KpnI
...-2126.. GGTACCAAG GCCGTGCCAA GAACCAATCA
-2097 GCGATGACTC TGGATTCTTG GGGGCTCCTC AGAGGCTCCG CAGCGGTGGA AGGACTGGAG
-2037 CTGCTGGCTG CCTCCTCCGG TGCAGCCTGT ATCCAGGTGC AGCGGCACTG GGGACGCGGT
-1977 GCATATCCCT TTGCTCAGAC TGCCACTGTG ACCCTTGCGC GAATATGTCC GGAGCCTAGA
-1917 AAGTGGTCCT GAGCGGCAGA GGGCCACTGG GTGGGTGGGT GAGTGGGGAG GTTCGGAAGG
-1857 CAGGACTGAG GGTAGGAAGC GAATTGGGCG ACTTGCTTCT CACGTTTGTC CCAATTAGCA
Krox24
-1797 CCAAGTTGGC AGACAATCCA CAAATCCACG ACACCTTTGC TTGTCCGCCC CCGCCGCCTC
-1737 CCTTCCCCCT CCCGTCAAAT TCTCTGCATT TCAGAATATT CTGACTGTAA GAAAACTTCA
-1677 GCTCCCCCTC CTCGGTTCAA GTGAAACAGA CACTTGGCAC CACCTTCTCC CAGTAACACT
NeuroD PstI
-1617 GTGTGGATGA ATCCCGGAGA TCCTCAACTG CAGCGCTTAC CAAACTGCAC CCTTTGGAGT
-1557 GTGGCTGGCG TGGGGTTGGG GGGCTAGGGT GGGCTGAGCA GAGAGGCCTC TGGGGGAGAG
-1497 GCGGGGTGGC SCGCGGAGGA GCGAGATGCA GGAGCAAGAT CGCCTGTGTG ACTGCATCTA
-1437 AAGCAGACTG GTCCAAGGAG TTCTGCCAGG GACACCACCG CCTGCATTGC GTCGGACCTG
-1377 ACCATCTCCA ATGTGAAATT CCCGGGGAAG GTCGCTCGCA ACGGAGGGCC GTGCGTGGGA
-1317 GGGGCTGCCG GCCCCCGGG AAGAGGAGTT GGTGGTGGGC TTTTCGGGGG GGGGGGCGGA
-1257 AGAAAATCTG TGCAGACAAG GATGGGAACT CTGAATGACA ACCCCCCTCT GCCAAACCAC
-1197 CCCCTCATAT TTTCCATCTA CCTCCTCGCT CCTGCCCTCC CCCGCCCTCC CCAACCCACG
-1137 CCCGGGTGGG CCAATCGCTG CTCCGCATTC CAGGCGCTTT CTCAGGTTTC TGCTGATCTT
<_____465_____>
NotI
-1077 GCAGCGCCCA GAAATGGACC GAGCGAACCT GCGGCCGCAC GCTCCCTGCC CCACTCGGAG
-1017 CTGCTGAAGG CTCGCGGCGC GCCGCGGAGA GAGGTCCGTT CTGTGGTAGC GCAGGTTACC
<_____
-957 GGAGAGCCAT GTAGAGCCAG GCTTTGAGCC GCCCGCCTCC CTCGGGATCG AATCAAGGCT
886_____>
-897 CCCGTAGTGT TCAGAGGGGG GCGAGAGTGC AGCTTATCGC CATTTGCTTG GGTGATTCCG
-837 GAGAAGGTGG CCTTTTTGTC TTTCCACCCC GCATCCTCCG GGACTCCCTT CCCCAGAGAG
-777 AGGACAGCGT CTCCAGGTTG CTTGCACCGT GTGAGGATGG GGGTAGGGTA CGGACGTTTC

-717 CTCCGTAGCT GCTCGATAAA ACGATCGGAC TCCTGAGAGG AGAAGGACGG GAAACGCGAG

-657 GGGCCCCGGA GCCTCTCGCA CGCTGCTGCG GTGGTGGCGG CTGCTCGTTC CGGCTGCTGC

-597 AAAGGCGACC TGCCGCATTC AGACTCTCCG CCGACTCAGC ACAGCCTCTG CGCCAAGCCG

-537 GCCCGCCAGC GTCCATCGAT CGCCCCGGTG GGAGCTTAGA AGGCGGCAGG CGAAGAGGGG

-477 TAGGAGGGGG GGAGAGCCAA GGAGCAGCAA AGTGGGTGAC AGGCCTGGGG AGCTGCTGAG

-417 TTGGCACTGC ACCGTGTCCT AGAAGGGCTG CAGGAGGGGA GGGGAGCCGG GGCGAGCCCC

-357 GGAAGCGATG GCAGGTGTGC ATTAAGGTGA TCAGAGCAGG AAGAAAAACC CATTCACCAA

HindIII

-297 CACAAAAACA GAGCACCCCC CCCAAAAAAT ACCCCAAGAG TAGCAAAAAA GCTTCACTGG

-237 GGGCCCTCAG GCGAGAGGGG TTTCCAAATT GCGTAAAAGG CAGAGTGGAG GAGGGGAGGT

-177 GATAACTAGC AAAGTTGCAG ACTTCTGAAC CTTCTGCTCT GAAGCCCCTC CCTGTGAGCG

-117 TGGGGAAGAC TCACTTTCCG GTGGGGGGGC CGCTTGGGTC CCCCCCACCC CTACTCCCTC

SmaI

-57 GCTCCTTCAC AACCCGGGCT CTCTCCTGGC CTCCTACCCC TGCACCCTGC TTTCATC**ATG** +3

Fig. 5. Analysis of proximal promoter region of Kv1.1 genomic clone. Reporter gene constructs indicated on left side of figure were used to transfect C6 glioma cells. The small shaded box indicated in the constructs represents the position and presence of the enhancer element of the parent vector. Following transfection cells were incubated with or without 8-cpt-cAMP.

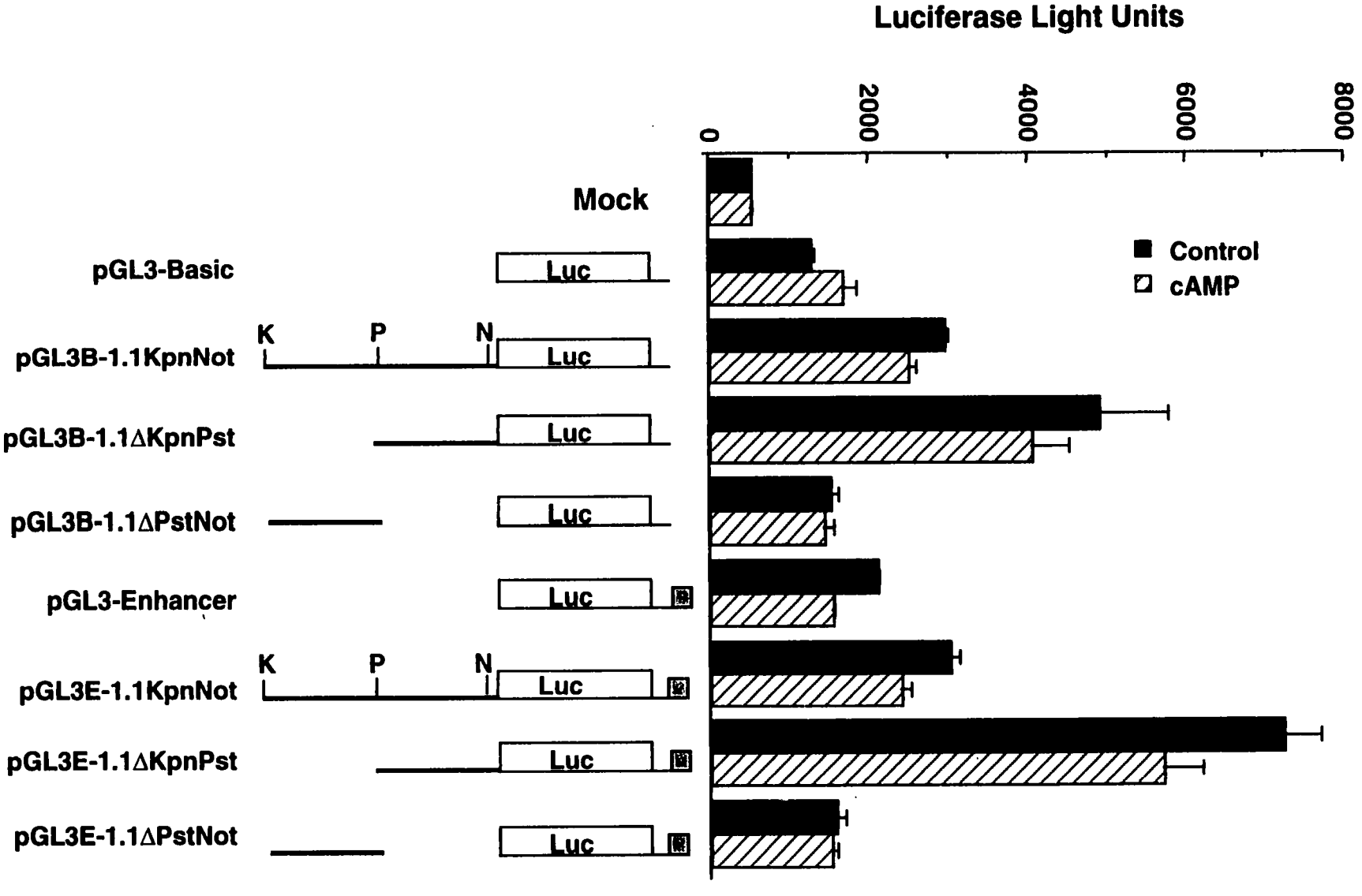
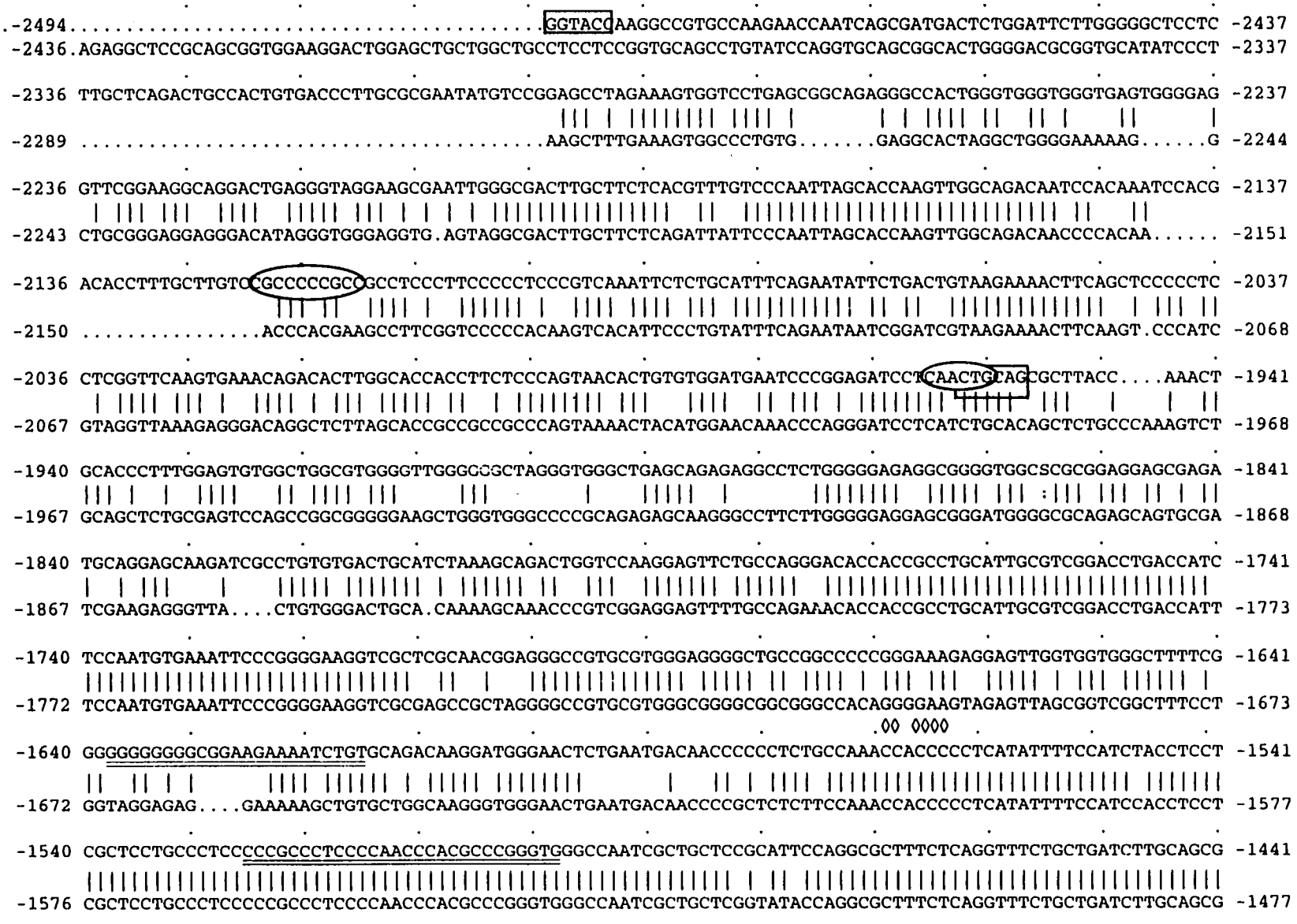
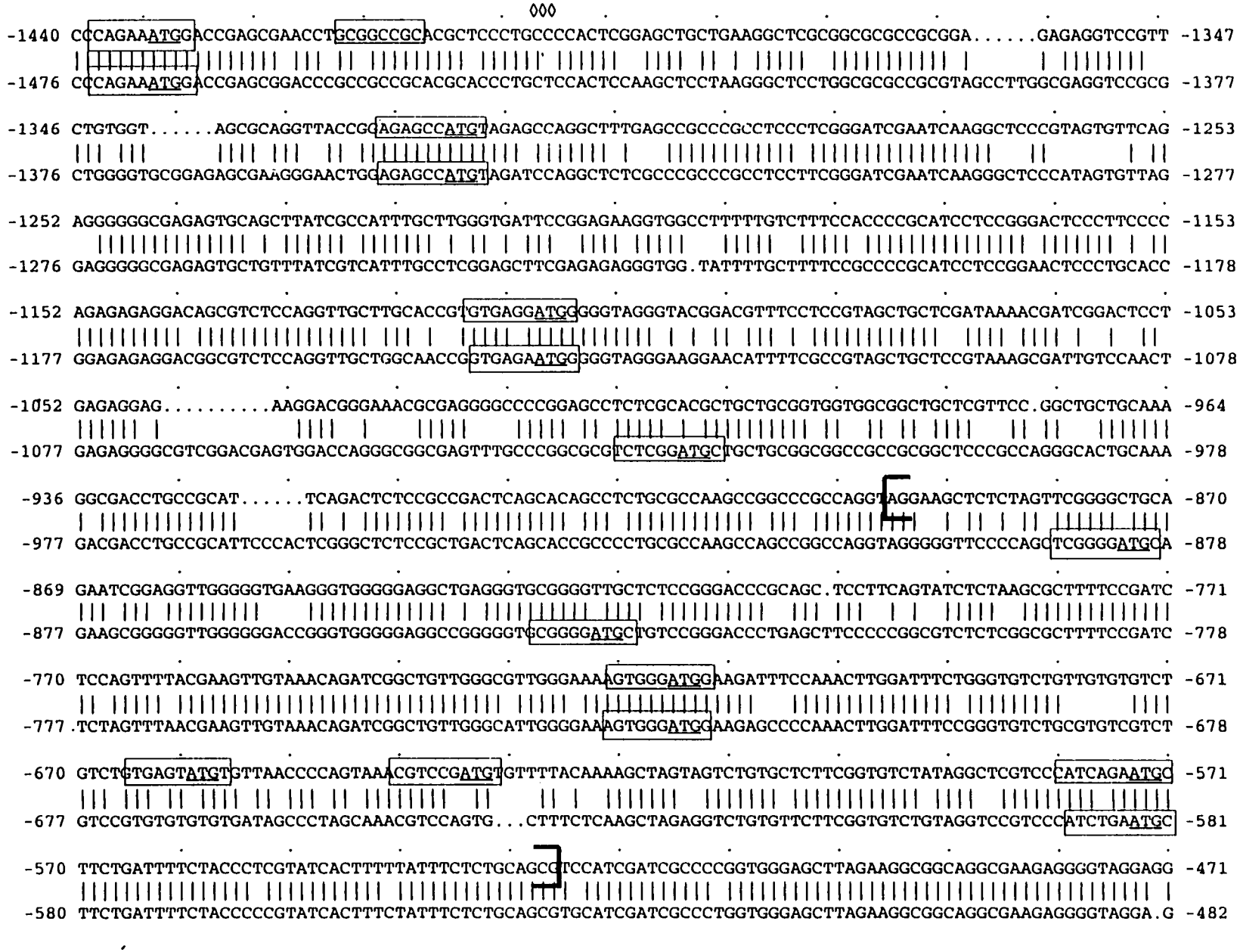

Luciferase Light Units
0
2000
4000
6000
8000
Mock
pGL3-Basic
pGL3B-1.1KpnNot
pGL3B-1.1ΔKpnPst
pGL3B-1.1ΔPstNot
pGL3-Enhancer
pGL3E-1.1KpnNot
pGL3E-1.1ΔKpnPst
pGL3E-1.1ΔPstNot
Luc
K
P
N
Control
cAMP

Fig. 6. Sequence alignment of mKv1.1 and hKv1.1 sequence. mKv1.1 (top strand) and hKv1.1 (bottom strand) sequences were aligned using the GAP subroutine of GCG. Transcription factor consensus site for Krox24 indicated by shaded ellipses. Regions of Sp1 site clusters indicated by double underline. Transcriptional starts indicated by "◊" above the bases mapped by S1 nuclease analysis. Restriction sites for KpnI, PstI, NotI, HindIII and SmaI are indicated by lightly shaded gray boxes. Intron indicated by shaded sequence between dark '[ ]'. Consensus Kozak translational starts of uORFs are boxed with ATG underlined. ORF translational start **ATG** indicated in bold and underlined.

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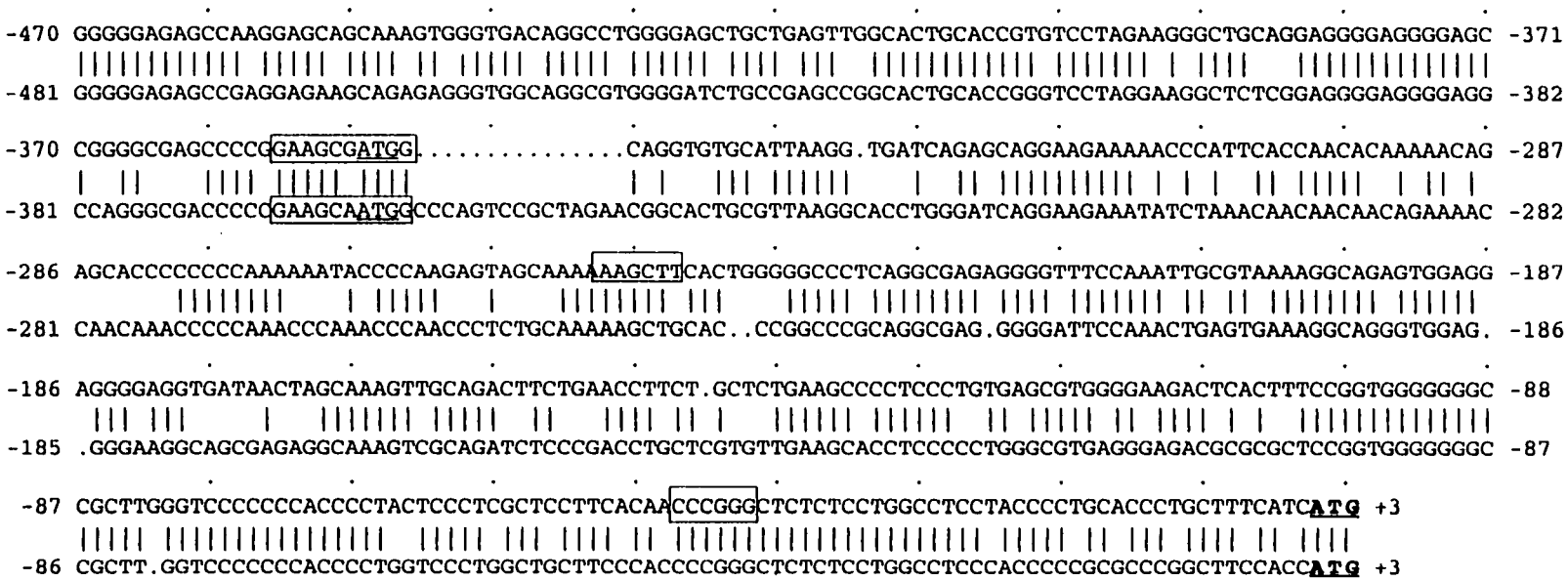
-1476 CCCAGAAATGGACCGAGCGGACCCGCCGCCGCACGCACCCTGCTCCACTCCAAGCTCCTAAGGGCTCCTGGCGCGCCGCGTAGCCTTGGCGAGGTCCGCG -1377
-1346 CTGTGGT.....AGCGCAGGTTACCGGAGAGCCATGTAGAGCCAGGCTTTGAGCCGCCCGCCTCCCTCGGGATCGAATCAAGGCTCCCGTAGTGTTCAG -1253
-1376 CTGGGGTGCGGAGAGCGAAGGGAACTGGAGAGCCATGTAGATCCAGGCTCTCGCCCGCCCGCCTCCTTCGGGATCGAATCAAGGGCTCCCATAGTGTTAG -1277
-1252 AGGGGGGCGAGAGTGCAGCTTATCGCCATTTGCTTGGGTGATTCCGGAGAAGGTGGCCTTTTTGTCTTTCCACCCCGCATCCTCCGGGACTCCCTTCCCC -1153
-1276 GAGGGGGCGAGAGTGCTGTTTATCGTCATTTGCCTCGGAGCTTCGAGAGAGGGTGG.TATTTTGCTTTTCCGCCCCGCATCCTCCGGAACTCCCTGCACC -1178
-1152 AGAGAGAGGACAGCGTCTCCAGGTTGCTTGCACCGTGTGAGGATGGGGGTAGGGTACGGACGTTTCCTCCGTAGCTGCTCGATAAAACGATCGGACTCCT -1053
-1177 GGAGAGAGGACGGCGTCTCCAGGTTGCTGGCAACCGGTGAGAATGGGGGTAGGGAAGGAACATTTTCGCCGTAGCTGCTCCGTAAAGCGATTGTCCAACT -1078
-1052 GAGAGGAG.....AAGGACGGGAAACGCGAGGGGCCCCGGAGCCTCTCGCACGCTGCTGCGGTGGTGGCGGCTGCTCGTTCC.GGCTGCTGCAAA -964
-1077 GAGAGGGGCGTCGGACGAGTGGACCAGGGCGGCGAGTTTGCCCGGCGCGTCTCGGATGCTGCTGCGGCGGCCGCCGCGGCTCCCGCCAGGGCACTGCAAA -978
-936 GGCGACCTGCCGCAT.....TCAGACTCTCCGCCGACTCAGCACAGCCTCTGCGCCAAGCCGGCCCGCCAGGTAGGAAGCTCTCTAGTTCGGGGCTGCA -870
-977 GACGACCTGCCGCATTCCCACTCGGGCTCTCCGCTGACTCAGCACCGCCCCTGCGCCAAGCCAGCCGGCCAGGTAGGGGGTTCCCCAGCTCGGGGATGCA -878
-869 GAATCGGAGGTTGGGGGTGAAGGGTGGGGGAGGCTGAGGGTGCGGGGTTGCTCTCCGGGACCCGCAGC.TCCTTCAGTATCTCTAAGCGCTTTTCCGATC -771
-877 GAAGCGGGGGTTGGGGGGACCGGGTGGGGGAGGCCGGGGGTGCGGGGATGCTGTCCGGGACCCTGAGCTTCCCCCGGCGTCTCTCGGCGCTTTTCCGATC -778
-770 TCCAGTTTTACGAAGTTGTAAACAGATCGGCTGTTGGGCGTTGGGAAAAGTGGGATGGAAGATTTCCAAACTTGGATTTCTGGGTGTCTGTTGTGTGTCT -671
-777 .TCTAGTTTAACGAAGTTGTAAACAGATCGGCTGTTGGGCATTGGGGAAAGTGGGATGGAAGAGCCCCAAACTTGGATTTCCGGGTGTCTGCGTGTCGTCT -678
-670 GTCTGTGAGTATGTGTTAACCCCAGTAAACGTCCGATGTGTTTTACAAAAGCTAGTAGTCTGTGCTCTTCGGTGTCTATAGGCTCGTCCCATCAGAATGC -571
-677 GTCCGTGTGTGTGTGATAGCCCTAGCAAACGTCCAGTG...CTTTCTCAAGCTAGAGGTCTGTGTTCTTCGGTGTCTGTAGGTCCGTCCCATCTGAATGC -581
-570 TTCTGATTTTCTACCCTCGTATCACTTTTTATTTCTCTGCAGCGTCCATCGATCGCCCCGGTGGGAGCTTAGAAGGCGGCAGGCGAAGAGGGGTAGGAGG -471
-580 TTCTGATTTTCTACCCCCGTATCACTTTCTATTTCTCTGCAGCGTGCATCGATCGCCCTGGTGGGAGCTTAGAAGGCGGCAGGCGAAGAGGGGTAGGA.G -482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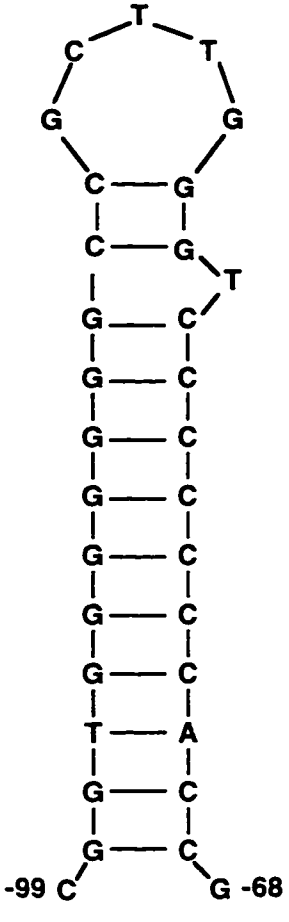
-481 GGGGGAGAGCCGAGGAGAAGCAGAGAGGGTGGCAGGCGTGGGGATCTGCCGAGCCGGCACTGCACCGGGTCCTAGGAAGGCTCTCGGAGGGGAGGGGAGG -382
-370 CGGGGCGAGCCCCGGAAGCGATGG.....CAGGTGTGCATTAAGG.TGATCAGAGCAGGAAGAAAAACCCATTCACCAACACAAAAACAG -287
-381 CCAGGGCGACCCCCGAAGCAATGGCCCAGTCCGCTAGAACGGCACTGCGTTAAGGCACCTGGGATCAGGAAGAAATATCTAAACAACAACAACAGAAAAC -282
-286 AGCACCCCCCCCCAAAAAATACCCCAAGAGTAGCAAAAAAGCTTCACTGGGGGCCCTCAGGCGAGAGGGGTTTCCAAATTGCGTAAAAGGCAGAGTGGAGG -187
-281 CAACAAACCCCCAAACCCAAACCCAACCCTCTGCAAAAAGCTGCAC..CCGGCCCGCAGGCGAG.GGGGATTCCAAACTGAGTGAAAGGCAGGGTGGAG. -186
-186 AGGGGAGGTGATAACTAGCAAAGTTGCAGACTTCTGAACCTTCT.GCTCTGAAGCCCCTCCCTGTGAGCGTGGGGAAGACTCACTTTCCGGTGGGGGGGC -88
-185 .GGGAAGGCAGCGAGAGGCAAAGTCGCAGATCTCCCGACCTGCTCGTGTTGAAGCACCTCCCCCTGGGCGTGAGGGAGACGCGCGCTCCGGTGGGGGGGC -87
-87 CGCTTGGGTCCCCCCCACCCCTACTCCCTCGCTCCTTCACAACCCGGGCTCTCTCCTGGCCTCCTACCCCTGCACCCTGCTTTCATCATG +3
-86 CGCTT.GGTCCCCCCCCACCCCTGGTCCCTGGCTGCTTCCCACCCCGGGCTCTCTCCTGGCCTCCCACCCCCGCGCCCGGCTTCCACCATG +3

Fig. 7. Representative structure of stem-loop conserved between species in Kv1.1 5'UTR. Structure based on prediction of MFOLD sub-routine of GCG program.


T
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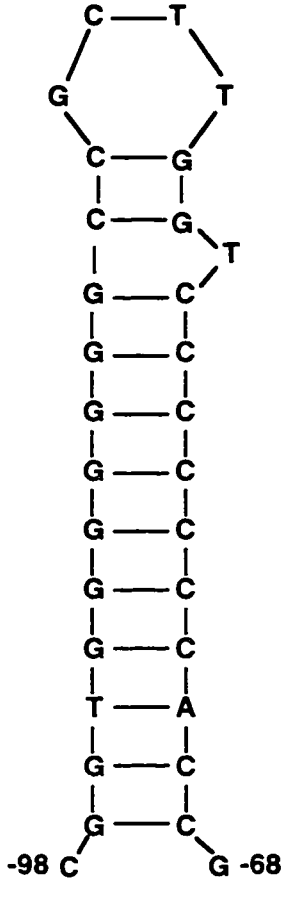
-99 C
G -68

mKv1.1


C
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G
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-98 C
G -68

hKv1.1

Fig. 8. Schematic representation of homology of Kv1.1 transcript between mouse and human. Note that the overall conservation in the 5'UTR is 78%, indicated by the narrow cross hatch regions. There are several regions with homology of >90% which is indicated by dark stippling.

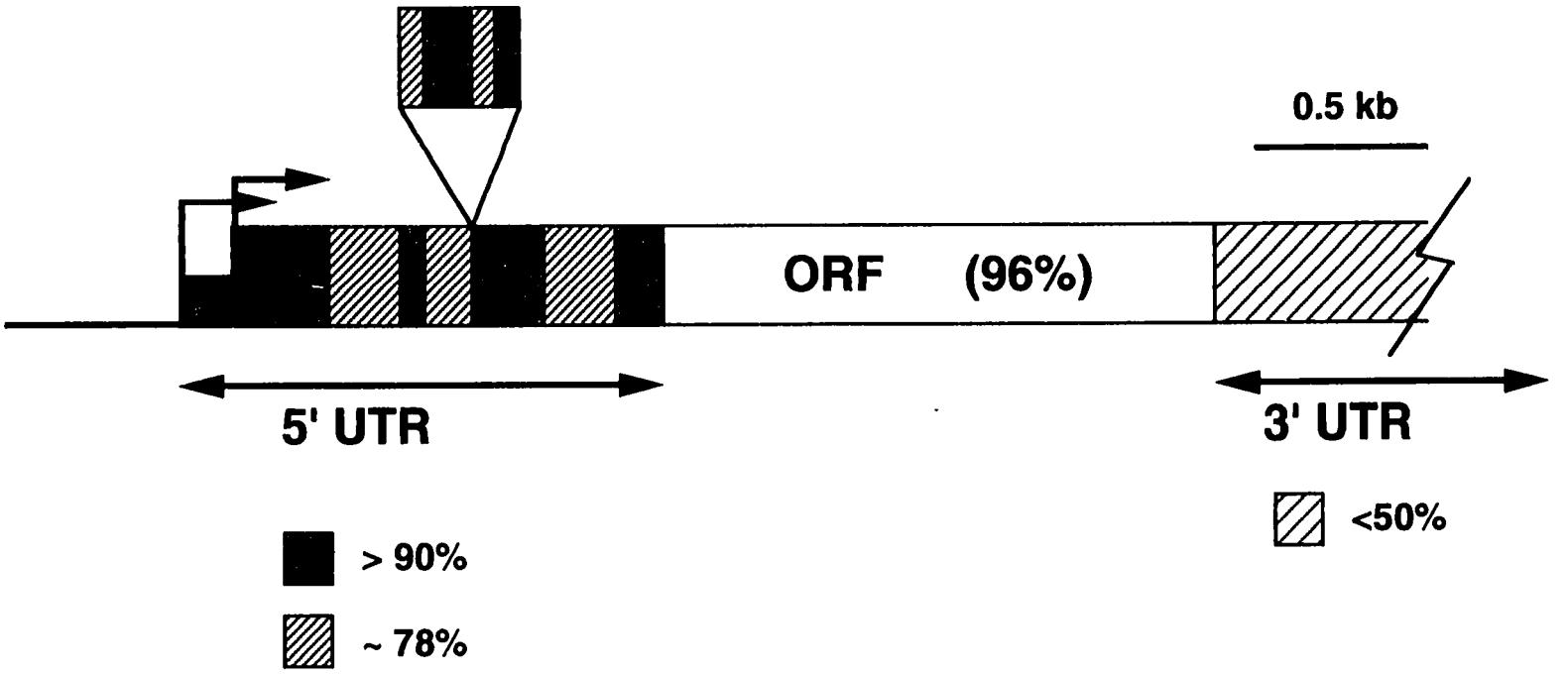

0.5 kb
ORF (96%)
5' UTR
3' UTR
> 90%
~ 78%
<50%

Table 1. List of uORFs **mKv1.1**

Position	nts	aa	GCC$^{A}/_{G}$CCATGG	Kozak %	seq. of peptide
-350	16	5	GAAGCGATGG	18.5%	MAGVH•
-574(I)	153	51	ATCAGAATGC	9%	MLLIFYPRITFYFSAASIDRPGGSLEGGRRR GVGGESQGAAKWVTGLGSC•
-635(I)	237	79	CGTCCGATGT	0.6%	MCFTKASSLCSSVSIGSSHQNASDFLPSYH FLFLCSVHRSPRWELRRRQAKRRRG GEPRSSKVGDRPGELLSWCTVS•
-660(I)	6	2	GTGAGTATGT	10%	MC•
-716(I)	51	17	AGTGGGATGG	18.5%	MEDFQTWISGCLLCVCL•
-1110	57	19	GTGAGGATGG	25%	MGVGYGRFLRSCSIKRSDDS•
-1318	3	1	AGAGCCATGT	3.9%	M•
-1433	51	17	CCAGAAATGG	18.5%	MDRANLRPHAPCPTRSC•

Table 1. List of uORFs **hKv1.1**

Position	nts	aa	GCC^/$_G$CCATGG	Kozak %	seq. of peptide
-361	31	10	GAAGCAATGG	18.5%	MAQSARTALR•
-584(I)	237	79	ATCTGAATGC	0%	MLLIFYPVSLSISLQRASIALVGA•
-723(I)	375	126	AGTGGGATGG	18.5%	MEEPQTWISGCLRVVCPCVCDSPSKRPVLS QARGLCSSVSVGPSHLNASDFLPRITFYFS AACIDRPGGSLEGGRRRGVGGRAEEKQRG WQAWGSAEPALHRVLGRLSEGRGGQGDPRS NGPVR•
-830(I)	60	20	GCGGGGATGC	6.7%	MLSGTLSFPRRLSALFRSLV•
-882(I)	123	41	TCGGGGATGC	6.7%	MQKRGLGGPGGGGRGCGDAVRDPELPPASL GAFPISSLTKL•
-1022	252	84	TCTCGGATGC	0.6%	MLLRRPPRLPPGHCKDDLPHSHSGSPL TQHRPCAKPAGQVGGSPARGCRSGGW GDRVGEAGGAGMLSGTLSFPRRLSALF RSLV•
-1135	57	19	GTGAGAATGG	25%	MGVGKEHFRRSCSVKRLSN•
-1342	3	1	AGAGCCATGT	3.9%	M•
-1469	51	17	CCAGAAATGG	18.5%	MDRADPPPGAPCSTPSS•

CHAPTER 2: ANALYSIS OF INTRON IN 5'UTR OF KV1.1 TRANSCRIPT

INTRODUCTION

In the original report of mKv1.1, sequence analysis of four independent picks of the initial cDNA clones of mKv1.1 revealed two with and two without an insert of 369bp in the 5'UTR region (Tempel et al., 1988). There are potential, weak splicing donor and acceptor sites at the 5' and 3' borders of this sequence. This putative intron is one of several aspects of the Kv1.1 transcript which indicates that it may be translationally regulated. One of the most striking of these is the structure of the transcript which consists of an intronless ORF of 1.5kb in a transcript of ~8kb. Baumann et al. (1988) cloned the rat Kv1.1 cDNA and reported 2.1kb of 3'UTR sequence. In the previous chapter the transcriptional start sites were set giving 1kb (1.3kb with intron sequence) of 5'UTR for mouse Kv1.1. Thus, the ~ 3kb of sequence that is unaccounted for in this ~8kb transcript is likely to be 3'UTR. The long 5'UTR region of the Kv1.1 transcript classifies it as one of the only 5 - 10% of eukaryotic mRNAs so far identified with long (600 - 1200bp) 5'UTRs (Jackson et al. 1990). Additionally, as seen in the previous chapter there is very high conservation between species of the 5'UTR, but not the 3'UTR, of Kv1.1. There are multiple uORFs in the 5'UTR of both mKv1.1 and hKv1.1 with several of the start sites for these contained within the putative intron region

(see Fig. 6, Chapter 1). These features suggest that Kv1.1 may be translationally regulated. Experiments were conducted to determine if the putative intron in the 5'UTR region represents two transcripts of differing translational regulation or if the presence of this putative intron in 50% of the original cDNA clones represents unprocessed heteronuclear RNA.

METHODS

Reagents

Cell culture media was purchased from GIBCO/BRL. Serum for cell culture was purchased from HyClone. Oligo-dT cellulose was purchased from Collaborative Research. ^{32}P-radionucleotides were purchased from New England Nuclear. Molecular biology reagents were purchased from Boehringer Mannheim Biochemicals. Other standard biochemical reagents were purchased from Sigma.

RPA Analysis:

RNA was extracted from the cells using guanidininum as described (Chomczynski and Sacchi, 1987). For RNase Protection Assay (RPA) varying amounts of either of total or Poly-A$^+$ selected RNA was incubated ^{32}P-radiolabeled riboprobes (specifics detailed in figure legends). The RPA protocol is that of (Bordonara, 1994) except that riboprobes were synthesized with 0.4μM UTP in the reaction mixture. In the hybridization, 5 x 10^5 cpm of

riboprobe was included per sample. The dried gels were exposed to a phosphorimager screen for quantitative analysis. The 5'Kv1.1 riboprobe was as previously described (Bosma et al., 1993). Riboprobe PP192 spanned the region PvuI to PstI surrounding the 5' splice site and was subcloned into the EcoRV to PstI sites of pBluescript vector. Riboprobe PH216 spanned the region of PstI to HpaI and was subcloned into the pBluescript vector at PstI to EcoRV. Riboprobe template for β-actin was purchased form Ambion.

Polysome analysis

Preparation of brain tissue for polysome analysis was according to the protocol of Garrett at al. (1989). HMN buffer composition: 20mM HEPES, pH 7.45, 100mM NaCl, 1.5mM $MgCl_2$. Sucrose gradients (sucrose prepared in HMN + 1% Triton X-100 + 3mM cycloheximide (CHX)) were prepared the afternoon prior to running the experiment by overlaying 5.5ml of 15% sucrose over 5.5 ml of 50% sucrose, tightly capping and laying the tubes horizontally at 4°C until needed. Extracts of mouse brain were prepared by quickly removing the brains and dissecting the cerebral hemisphere and cerebellum from five mice. The tissue was homogenized in buffer HMN + 1% Triton X-100 + 3mM CHX + 5 mM ribonucleotide complex. These extracts were placed in silanized 15ml corex tubes and centrifuged 5 minutes at 10,00x*g*, Sorvall SA600 rotor. The supernatants were transferred to clean tubes with 100µl heparin/ml extract (10mg heparin/ml HMN). The sucrose

gradients were carefully placed vertically and 0.5ml of extract carefully overlaid. Gradients were run for 1hour 50minutes, 36,000rpm Beckman SW40 rotor. Fractions (1ml) were collected on an Isco density gradient fraction collector (model 640), 0.1ml 10% SDS and Proteinase K to 200μg/ml were added followed by incubation at 42°C for an hour. The fractions were phenol extracted and the RNA precipitated in the presence of glycogen and tRNA. The pellet was resuspended in 300μl GuiSCN solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol) and transferred to a 1.7ml eppendorf and precipitated with 2.5 vol. EtOH. The final pellet was resuspended in 0.1ml 0.1% SDS. RPA analysis was conducted on 20μl of each fraction as described above.

RESULTS

RPA analysis of putative intron

To determine if the 369bp of the 5'UTR is a fully spliced intron or if it represents two species of the mRNA, we conducted RPA analysis with riboprobes designed to 1) span the putative 5' splice site (PP192), and 2) span a region entirely within the putative intron (PH216). Results from these assays showed that in total mRNA made from mouse cerebrum and hindbrain+midbrain the ratio of the signal intensity of the region protected by the full length probe (unspliced) relative to the signal of the shorter fragment

(spliced) protected by the same probe was 8 - 9% (Fig. 9). In contrast, in mRNA made from cerebellum the ratio of full-length probe/partial probe signal intensity was approximately 3 times higher (21-22%) (Fig. 9). To determine if this difference was due to the presence in the cerebellum of a larger pool of unprocessed mRNA, poly-A^+ mRNA was made and the RPA repeated. As can be seen in Fig. 10, the full-length protected fragment is virtually undetectable in the poly-A^+ mRNA made from forebrain but remains at a high level in the poly-A^+ mRNA made from cerebellum. This result is supported by the protection pattern with a riboprobe (PH216) that protects entirely within this putative intron (Fig. 10) This suggests that there may be two species of mature mRNA for mKv1.1 in the cerebellum.

Polysome analysis of putative intron

To determine if the longer, intron containing transcript is translated, we conducted polysome analysis on extracts of mouse brain cerebellum and cerebrum. RPA analysis with the 5'Kv1.1, PP192, and β-actin riboprobes was done on each of the fractions from the sucrose gradient. The β-actin riboprobe was used as a positive control demonstrating that the gradients separated polysomes and to show the position of a highly loaded transcript. As can be seen in Fig. 11, β-actin is located in peaks in fractions 7, 8 and 9 which correlates with mRNAs loaded equal to or greater than 5- or 6-

ribosomes/transcript. To demonstrate that we could detect Kv1.1 loaded on the gradients, we used the 5'Kv1.1 riboprobe and demonstrated that this transcript was loaded and peaks at fractions 5, 6 and 7, slightly and consistently lower than β-actin (Fig. 11). Using probe PP192, which spans the 5' splice site, we detected both the spliced and intron containing species very cleanly in the lane corresponding to total cerebellar RNA (Fig. 11 compare lane C to lane F in PP192 panel). In the gradient fractions however, on films where the protected band corresponding to the spliced transcript is over-exposed we detect a low level of the intron containing species in nearly all lanes, though it is a higher intensity in lanes 2 and 3 (cerebellum) and lane 3 (forebrain). These lanes (2 and 3) corresponds to the monosome fractions. Note also that in the gradient fractions (as compared to tRNA and total RNA lanes, t, F and C) the probe is incompletely digested. This pattern of splicing in the various sucrose fractions was seen in two completely separate experiments with different animals. We conclude from these data that the transcript species which contains the 'intron' is not translated.

DISCUSSION

The presence of a putative intron in the 5'UTR in 50% of the original cloned cDNAs suggested that either there were two different mature transcript species for Kv1.1 or that this represented incomplete processing of

the nuclear transcript. Our initial RPA analysis did not definitively distinguish between these two possibilities. Polysome analysis indicates that the intron containing species is not translated. We therefore conclude that this represents incomplete nuclear processing of the Kv1.1 transcript.

Fig. 9. RPA analysis of total RNA to determine presence of 5'UTR intron. The location of the riboprobes used is indicated on the schematic map. Total RNA from the indicated brain regions were used with each probe. The spliced and unspliced protected fragments of probe PP192 are indicated.

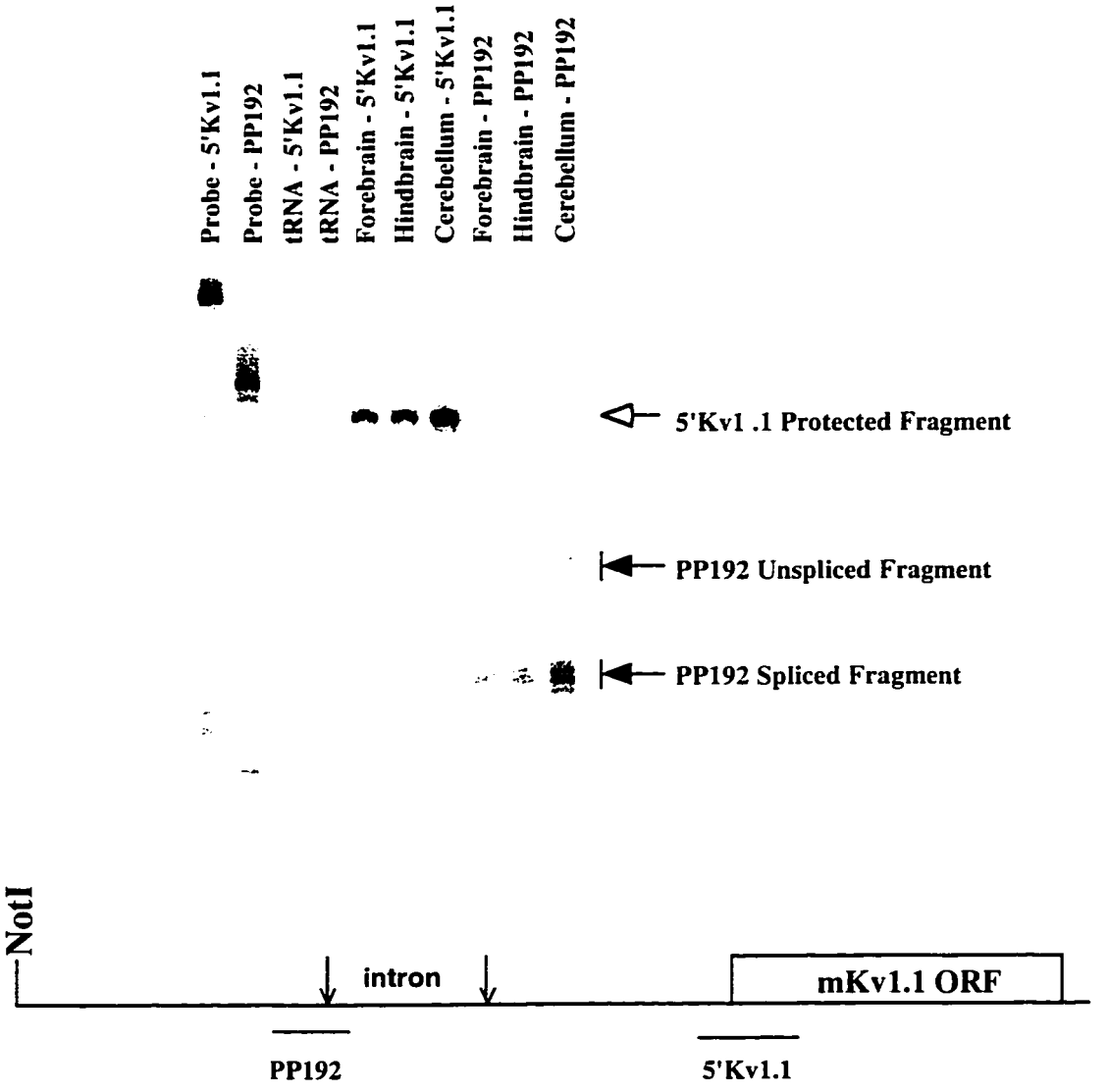

Probe - 5'Kv1.1
Probe - PP192
tRNA - 5'Kv1.1
tRNA - PP192
Forebrain - 5'Kv1.1
Hindbrain - 5'Kv1.1
Cerebellum - 5'Kv1.1
Forebrain - PP192
Hindbrain - PP192
Cerebellum - PP192
5'Kv1 .1 Protected Fragment
PP192 Unspliced Fragment
PP192 Spliced Fragment
NotI
intron
mKv1.1 ORF
PP192
5'Kv1.1

Fig. 10. RPA analysis of poly-A^+ RNA to determine presence of 5'UTR intron. The location of the riboprobes used is indicated on the schematic map at the bottom of the figure. Each probe was hybridized with total, poly-A^- and poly-A^+ RNA prepared from mouse forebrain and cerebellum.

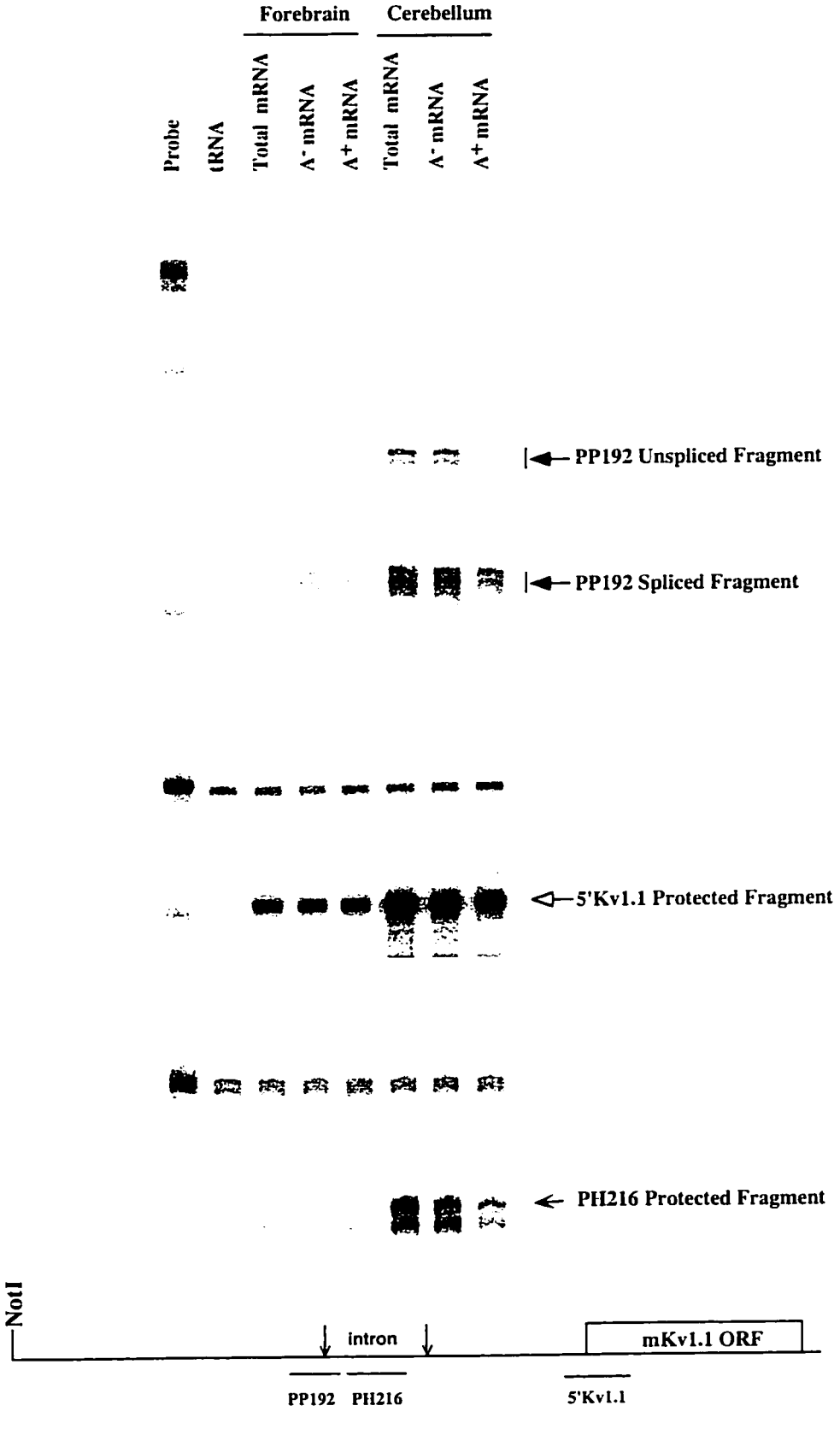
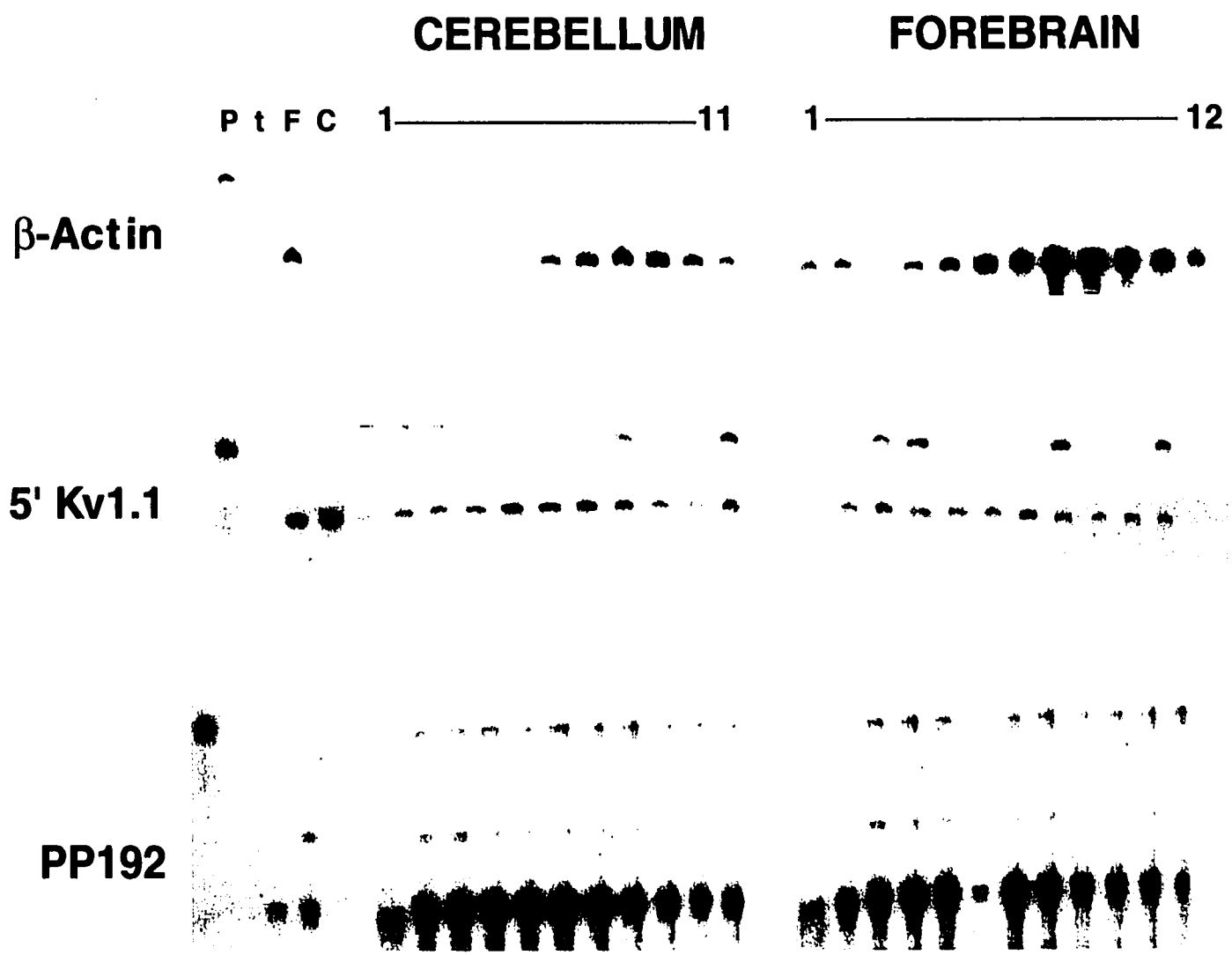

Forebrain
Cerebellum
Probe
tRNA
Total mRNA
A⁻ mRNA
A⁺ mRNA
Total mRNA
A⁻ mRNA
A⁺ mRNA
PP192 Unspliced Fragment
PP192 Spliced Fragment
5'Kv1.1 Protected Fragment
PH216 Protected Fragment
NotI
intron
mKv1.1 ORF
PP192
PH216
5'Kv1.1

Fig. 11. Polysome analysis to determine presence of 5'UTR intron. Polysome fractions were subjected to RPA analysis with the riboprobes indicated to the left of each panel. Fraction 1 is the top of the gradient and fractions 11 (cerebellum) or 12 (forebrain) are the bottom of the gradients. Lane P indicates the respective Probes, t indicates tRNA, F indicates total RNA from mouse forebrain, C indicates total RNA from mouse cerebellum.


CEREBELLUM
FOREBRAIN
P t F C
1
11
1
12
β-Actin
5' Kv1.1
PP192

CHAPTER 3: CYCLIC-AMP REGULATES POTASSIUM CHANNEL EXPRESSION IN C6 GLIOMA BY DESTABILIZING Kv1.1 mRNA

INTRODUCTION

Regulation of gene expression can occur at the level of transcription, translation, or post-translationally. Among the 20 different genes encoding voltage-gated potassium (Kv) channels, transcriptional regulation has been analyzed for the Kv1.5 and Kv3.1 genes (Gan et al., 1996; Levitan et al., 1995; Mori et al., 1995; Mori et al., 1993; Takimoto and Levitan, 1996). For example, Takimoto et al. (1993) demonstrated that expression levels of Kv1.5 were affected at the level of transcription with dexamethasone treatment but that mRNA half-life and protein turn-over were unchanged. Post-translational regulation has also been convincingly demonstrated for a number of Kv channels. For example, phosphorylation by tyrosine kinase, protein kinase C and protein kinase A have each been shown to modulate Kv channel function (Chung and Schlichter, 1997; Holmes et al., 1996; Huang et al., 1993) and a channel-associated β-subunit (Kvβ2) has been shown to promote translocation of Kv1.2 to the plasma membrane (Shi et al., 1996). In contrast, relatively little is known about regulation of Kv genes at the translational level. Given the fact that many Kv transcripts are large (6 to 12kb) but contain

relatively small open reading frames (1.5 to 2.5kb), it seems likely that the untranslated portions of these transcripts may have a regulatory function.

Regulation of gene expression can occur at the level of transcription, translation, or post-translationally. Among the genes encoding voltage-gated potassium (Kv) channels, transcriptional regulation has been analyzed for Kv1.5 and Kv3.1 (Gan et al., 1996; Levitan et al., 1995; Mori et al., 1995; Mori et al., 1993; Takimoto and Levitan, 1996). For example, Takimoto et al. (1993) demonstrated that expression levels of Kv1.5 were affected at the level of transcription with dexamethasone treatment but that mRNA half-life and protein turn-over were unchanged. Post-translational regulation has also been convincingly demonstrated for a number of Kv channels. For example, phosphorylation by tyrosine kinase, protein kinase C and protein kinase A have each been shown to modulate Kv channel function (Chung and Schlichter, 1997; Holmes et al., 1996; Huang et al., 1993) and a channel-associated β-subunit (Kvβ2) has been shown to promote translocation of Kv1.2 to the plasma membrane (Shi et al., 1996). In contrast, relatively little is known about regulation of Kv genes at the translational level. Given the fact that many Kv transcripts are large (6 to 12kb) but contain relatively small open reading frames (1.5 to 2.5kb), it seems likely that the untranslated portions of these transcripts may have a regulatory function.

There exist a variety of forms of translational regulation - including changes in nuclear processing of the transcript (splicing, capping, polyadenylation, and nuclear export) as well as changes in the efficiency of translation (ribosome binding, loading, scanning, and initiation) and changes in transcript stability (Mathews et al., 1996). Well studied examples include S-adenosylmethionine decarboxylase, regulated by changing the efficiency of ribosome loading and scanning (Hill and Morris, 1992); iron metabolism, regulated by blockade of ribosome scanning and by regulation of transcript stability in the ferritin and transferrin receptor genes, respectively (Harford and Klausner, 1990). Among ion channel genes, Wymore et al. demonstrated that alternatively spliced 3′ untranslated regions (UTRs) from Kv1.4 cause differing levels of Kv1.3 current to be induced in *Xenopus* ooctyes injected with cRNA from the chimeric constructs, suggesting that differences in the sequence of the alternative 3'UTRs result in different translational efficiencies.

We have used the C6 glioma cell line to study the molecular mechanisms regulating the endogenously expressed Kv1.1 potassium channel. We show that when intracellular cAMP levels are increased there is a rapid reduction in the steady state level of Kv1.1 RNA but only a small decrease in the transcription rate of the Kv1.1 gene. The decrease in the steady state abundance of the Kv1.1 transcript is followed by a decrease in

Kv1.1 channel protein levels and a decrease in the whole cell potassium (K^+) current. Finally, we demonstrate that Kv1.1 is important in setting the resting membrane potential of C6 glioma cells. These data provide the first example of ion channel gene expression being regulated by cAMP via a destabilization of the RNA transcript.

METHODS

Reagents:

Tissue culture media, penicillin/streptomycin, and L-glutamine were purchased from GIBCO/BRL. Fetal bovine serum and equine serum were purchased from HyClone. Forskolin, and 1,9-dideoxy-forskolin were purchased from CalBiochem, template for rat-cyclophilin riboprobe from Ambion, radioisotopes from NEN, and recombinant RNasin® inhibitor from Promega. All other reagents were purchased from Sigma.

Cell Culture:

The C6 Glioma cell line was purchased from ATCC and maintained at less-than 80% confluence in F10 medium supplemented with 15% equine serum, 2.5% fetal bovine serum, 2 mM glutamine and 10U/ml penicillin G, 10μg/ml streptomycin sulfate. Cells between passages P41 and P50 were seeded at 2.0 X 10^4 cells/cm^2 except that a lower density was used for seeding cells used for electrophysiology (see below). Single-dose pharmacological treatments were

initiated 24 hours after seeding; cells were harvested at the time periods indicated in the figure legends. For all experiments vehicle was added to untreated control cells.

RNA Analysis:

RNA was extracted from the cells using guanidininum as described (Chomczynski and Sacchi, 1987). For RNase Protection Assay (RPA) 5ug of total RNA per sample was incubated with two ^{32}P-radiolabeled riboprobes; one for mouse Kv1.1 (Bosma et al., 1993) and one for rat cyclophilin. The RPA protocol is that of (Bordonara, 1994) except that Kv1.1 riboprobe was synthesized with 0.4μM UTP in the reaction mixture and cyclophilin with 20μM UTP in the reaction mixture. In the hybridization mixture 5×10^5 cpm of Kv1.1 and 1×10^5 cpm of cyclophilin were included per sample. The dried gels were exposed to a phosphorimager screen for quantitative analysis. The results are represented as the ratio of Kv1.1 protected fragment/cyclophilin protected fragment. Statistics were calculated using a two-tailed *t* test or a one-way ANOVA followed by Tukey's multiple comparison analysis as indicated in figure legends.

Nuclear Run-On:

To prepare nuclei for the nuclear run-on transcription assay 5×10^7 cells were quickly removed from the flasks and transferred to prechilled 50 ml conical tubes. The cells were pelleted and washed once with ice cold PBS. Nuclei

were prepared as described (Ausubel et al., 1991) except that 18 strokes of the homogenizer were required to obtain clean nuclei. The isolated nuclei were intact and clean of cytoplasmic debris as judged by microscopy with a 40X objective under phase contrast illumination. Nuclei were resuspended in 200µl glycerol storage buffer and nuclear run-on transcription reactions were started immediately by addition of 250µl 2X buffer with NTPs, 250 µCi (25µl) ^{32}P-UTP (3000 Ci/mmol) and 5µl RNase Inhibitor (20 - 40U/µl) (Ausubel et al., 1991). The reaction was allowed to proceed for 40 min. at 30°C at which point it was stopped by addition of RNase free DNase I followed by Proteinase K digestion as described (Ausubel et al., 1991). Then GuiSCN solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M β-mercaptoethanol) and 2M sodium acetate, pH 4.0 were added. This was followed by extraction with phenol (water saturated) and chloroform. The aqueous phase was precipitated overnight in 30ml silanized corex tubes (Fei and Drake, 1993). The RNA pellet was resuspended in 3ml of GuiSCN solution and reprecipitated. The RNA pellet was resuspended in 0.3M sodium acetate, pH 5.2, 0.1% SDS and reprecipitated, twice. The final pellet was resuspended in 2ml of hybridization solution (10mM TES, pH 74., 10mM EDTA, 0.3M NaCl, 5% SDS). The cmp/ml were calculated and adjusted to be nearly equal and denatured salmon sperm DNA was added to a final

concentration of 0.2mg/ml. This was hybridized to membrane bound probes (prehybridization at 60°C for 4 hours in hybridization solution with denatured salmon sperm DNA at 0.2mg/ml) at 60°C for approximately 42 hours using a Hybridization Incubator (Robbins Scientific). The blots were washed as described (Ausubel et al., 1991). Phosphorimager analysis was used for quantitation.

Slot Blot Preparation:

Probes were subcloned into M13mp18 and M13mp19 and single stranded DNA prepared. The following regions were sub-cloned: for EF-1alpha, the EcoR I fragment encompassing the entire ORF; for GAPDH the Pst I fragment encompassing 1.2kb of the ORF; for LDH the Bbs I to Xba I fragment from the pLDHA-5 plasmid (gift of R. Jungmann); for Kv1.1, a 370 bp region of unconserved 3' sequence including 160bp of 3'UTR. Using a slot-blot apparatus (BioRad) 5µg of ssDNA was bound per slot in 6X SSC then UV crosslinked (Stratagene).

Protein:

Following treatments, cells were washed twice with PBS and 1 ml of homogenization buffer (40mM TrisHCl, pH 8.0, 1µg/ml leupeptin, 1µg/ml pepstatin A, 15mM benzamidine) was added sequentially to 2 x 100mm petri dish. The cells were scraped from the bottom and the sample transferred to an ice cold dounce homogenizer and lysed with 25 strokes of the tight pestle.

The sample was transferred to 1.7ml eppendorf tube and centrifuged at 10,000g for 30 min. The supernatant (cytosolic fraction) was transferred to a clean tube and stored on ice. The pellet (membrane fraction) was resuspended in 1 ml homogenization buffer and homogenized and centrifuged as described (Sonnenburg et al., 1993). This second supernatant was discarded and the pellet was resuspended and rehomogenized in 1ml homogenization buffer, 0.8 ml were transferred to a 1.7 ml eppendorf tube and 0.2 ml of 5X Lamlei sample buffer added and the samples were boiled for 10 min. and stored at -80°C. The remaining 0.2ml of sample was saved for protein determination. For analysis, aliquots of samples were reboiled and 25μg/lane were run on 8% PAGE gels for 200V-hours and then transferred onto nitrocellulose at RT for 250V-hours. Following transfer, the blots were blocked for 1 to 4 hours at RT in 2.5% Blotto. Primary antibody, Rb anti-MK1 isotype specific (Wang et al., 1993), in 2.5% Blotto at 1/400 dilution was incubated for 2 hours at RT. Blots were washed 4 x 5 min. with TBS+0.1% Tween 20. Secondary antibody, Horseradish peroxidase conjugated Goat anti Rabbit IgG at 1/5000 in 2.5% Blotto, was incubated for 2 hours at RT. Blots were washed as described and incubated in substrate, Pierce ECL reagent and the blots exposed to film.

PKA Assay:

PKA activity was assayed as described (Graves et al., 1993). This assay quantitated the kinase activity in the extracts that resulted in incorporation of ^{32}P onto the peptide substrate, Kemptide, and could be inhibited by the PKA specific peptide inhibitor, PKI.

Electrophysiological measurements:

Cells were seeded at a low density ($<1.2 \times 10^4$ cells/ml) on small glass chips since cells may be connected by gap junction after proliferation and contact between cells. Cells were washed with Ringer's solution before the measurement of K currents. Single isolated cells were used for patch-clamp measurement (Hamill et al., 1981; Wang et al., 1992). Pipette capacitance was compensated before the formation of gigaseal. Membrane capacitance was measured by reading the value for slow capacitance after optimal compensation of leak and capacitance currents in whole-cell configuration. Voltage-dependent K currents were activated by depolarizing voltage steps. Current was amplified by an EPC-7 (List, Germany). Leak and capacitance currents were subtracted by using current recordings with negative voltage step. Current density was calculated by dividing current amplitude by the cell membrane capacitance. Resting membrane potential was measured in current-clamp mode ($I = 0$).

Cells were perfused with Ringer's solution of the following composition (in mM): 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. Pipette resistance was 2-3 MΩ when filled with intracellular solution: 140 KCl, 2 $MgCl_2$, 5 EGTA, and 10 HEPES, adjusted to pH 7.2 with KOH. For solutions containing dendrotoxin-I (DTX-I), 0.01% bovine serum albumin was added to reduce nonspecific binding. A multibarreled solution exchange system applied the toxin to cells. All data are expressed as the mean ± SEM.

RESULTS

In order to study the mechanism of the endogenous regulation of expression of Kv1.1 potassium channel, we screened neuronal (Neuro-2-A, NB41A3, N4, N18, SH-SY-5Y, IMR-32) and non-neuronal (CHO, AtT-20, MDCK, COS-7, C6 glioma) cell lines by ribonuclease protection assay (RPA) to find ones that expressed the Kv1.1 channel. We found that only C6 glioma expressed Kv1.1 in the unstimulated state, as previously reported by Wang et al. (1992). C6 glioma were treated with a single, continuous application of various pharmacological agents known to stimulate second-messenger systems and changes in the steady state levels of Kv1.1 mRNA were measured relative to cyclophilin mRNA. When the cells were treated with either the phorbol ester, PMA (100nM), or with elevated extracellular KCl

(50mM), the steady state level of Kv1.1 RNA was reduced to less than 50% of control levels when measured at either 6 or 24 hours (Table 3). When the cells were treated with the calcium ionophore A23187 (1μM) there was a biphasic change in the Kv1.1 steady state RNA level with about 40% of control remaining after 6 hours of treatment; this was followed by a rebound to ~130% of control after 24 hours of treatment (Table 3).

When the C6 Glioma were treated with 10μM (-)-isoproterenol + 500μM 3-isobutyl-1-methylxanthene (Iso/IBMX) to elevate intracellular cAMP concentrations the steady state level of Kv1.1 RNA decreased by ~80% (Fig. 12). Focusing on this robust change, we determined the time course of the decrease in control or Iso/IBMX treated cultures taking time points out to 72 hours of treatment. Kv1.1 RNA level was ~50% of control by 2 hours and showed maximal effect after 4 - 6 hours of treatment (Fig. 12B). Similar decreases were seen when cAMP levels were raised by use of forskolin (10μM) or the non-hydrolizable cAMP analog, 8-cpt-cAMP (100μM) (Table 3). Combined treatment with Iso/IBMX was necessary to attain the full decrease as Iso or IBMX alone after 6 hours showed levels of Kv1.1 RNA at 50% of control compared to 20% for Iso/IBMX (Table 3 and Fig. 13B). Garber et al. (1990) and Zerr et al. (1994) have demonstrated that forskolin can act as a direct blocker of K^+ currents. In light of these observations, we tested the

inactive forskolin analog, 1,9-dideoxy forskolin (10μM), and determined that it did not affect Kv1.1 RNA levels (Table 3). Therefore, we conclude that the effect of forskolin on decreasing Kv1.1 transcript levels was via activation of adenylyl cyclase.

To demonstrate that treatments with Iso/IBMX and forskolin were indeed elevating cAMP concentrations in the cells, we assayed Kv1.1 RNA levels and protein kinase A activity in paired cultures treated with the various agents for 6 hours. RNA was prepared from half of the cultures for RPA assays; protein was extracted from the remaining cultures for PKA activity assays. Fig. 13 shows that there was an inverse correlation between the PKA activity and the Kv1.1 steady state RNA level, suggesting that the decrease of the Kv1.1 transcript is linked to activation of PKA.

To examine the mechanism of regulation of Kv1.1 transcript levels by cAMP, we measured the Kv1.1 message half-life by treating parallel cultures with vehicle, Iso/IBMX, or the transcription inhibitor DRB (Fig. 14A). Cultures were harvested at several time points and RNA level was measured by RPA. Half-life was determined from semi-log plots of the data (Fig. 14A). In untreated control cells there was no change in the Kv1.1 transcript level whereas with the transcription inhibitor DRB the Kv1.1 RNA half-life was ≥6 hours and with Iso/IBMX half-life was <3 hours. Essentially identical results were obtained when transcription was inhibited with actinomycin D (data not

shown). These data indicate that increasing cAMP leads to an increased rate of degradation of Kv1.1 RNA, one that is more rapid than can be accounted for by turning off gene transcription with either of the classic transcription inhibitors DRB or actinomycin D.

Besides increased degradation, an alternative explanation for the decrease in Kv1.1 RNA with increasing cAMP might be that newly synthesized Kv1.1 RNA was blocked from nuclear export. This type of regulation has been observed for host transcripts in viral infected cells (Babich et al., 1983; Katze and Krug, 1984). To determine if increases in cAMP lead to a block of nuclear export of Kv1.1 RNA, we performed RPAs on the cytoplasmic RNA versus nuclear RNA prepared from C6 glioma treated for 6 hours with vehicle or Iso/IBMX. The Kv1.1 transcript level for Iso/IBMX compared to control treated cells was 19% in cytoplasmic RNA and 23% in nuclear RNA. These decreases are of the same magnitude as seen in total cellular RNA (Table 3; Fig. 12B; Fig. 13) suggesting that the decrease in Kv1.1 RNA was not due to a block of nuclear export.

In order to determine the effect of increased cAMP on transcription of the Kv1.1 gene we conducted nuclear run-on transcription assays. Transcription measured from nuclei prepared from cells treated for 6 hours demonstrates that there is a 35% decrease in Kv1.1 signal relative to that detected in untreated cells (Fig. 14B). As a positive control for the assay,

measurements of transcription of LDH made with the same nuclei showed an average 3.5-fold increase in transcription with Iso/IBMX treatment, as previously reported by Jungamann et al. (1983). These data suggest that increased cAMP causes an apparent decrease in Kv1.1 transcription. A caveat to this interpretation is that inherent to the measurements of nuclear run-on assays is the assumption that the stability of the newly transcribed RNA is unaffected by the pharmacological treatments. Given that we know Iso/IBMX treatment affects RNA stability (Fig. 14A), the apparent 35% reduction in newly synthesized Kv1.1 transcript measured by nuclear run-on assay may over-estimate the actual change in Kv1.1 transcription rate.

Schimke (1973) noted that, in general, tightly regulated proteins are encoded by RNAs that turn-over rapidly. Having determined that the Kv1.1 transcript was rapidly decreased when cAMP levels were elevated, we performed Western blot analysis on membrane fractions prepared from control or Iso/IBMX treated cells (Wang et al., 1993). As seen in Fig. 15, Kv1.1 protein levels in Iso/IBMX treated cells first show a noticeable decrease at 9 hours, with barely detectable levels at 48 hours.

To measure changes in density of voltage dependent K^+ channels in the plasma membrane, we conducted whole-cell patch-clamp experiments on control and Iso/IBMX treated cells. Potassium currents were activated by

voltage steps from a holding potential of -70 mV to different levels of depolarization. The currents started to activate around -40 mV, near the activation threshold for Kv1.1 (Bosma et al., 1993; Stuhmer et al., 1989). To compare channel density, K^+ current was elicited by a voltage step from -70mV to 0 mV (Fig. 16A). As some cells showed fast-inactivating currents at the beginning of depolarizing pulse (see also ref. Wang et al., 1992), the amplitude of sustained K^+ current was measured as the mean of current during the last 10 ms of 100 ms long pulse. (Kv1.1 expressed in CHO cells shows <5% inactivation after 500ms pulse (Bosma et al., 1993).) Because relatively high variability was observed in current density between cells whether treated or control, we performed a population study (Fig. 16B). Current density of control and treated groups were significantly different ($p \leq 0.01$, two tailed *t*-test) with prolonged Iso/IBMX treatment, 24 and 48 hrs. These differences were consistently observed in four different sets of cells.

When cells were acutely treated (3 min.) with Iso/IBMX, the amplitude of the K^+ current was not changed (100 ± 4 % of that before the treatment, n = 5). A similar lack of acute effect on K^+ current was observed when either PKI or PKA catalytic subunit was included in the patch pipette during whole-cell recordings of Kv1.1 transfected CHO cells (Bosma et al., 1993).

Kv1.1 is one of three Shaker family potassium channels (Kv1.1, Kv1.2 and Kv1.6) sensitive to dendrotoxin-I (DTX-I) found in the venom of black

mamba snakes (Pongs, 1992). C6 Glioma were treated with DTX-I to determine if the K^+ currents recorded in these cells were sensitive to this relatively specific Kv channel blocker. DTX-I (100nM) reduced K^+ currents significantly (6 ± 1 % of control, n = 14; Fig. 17A). Recovery from block by DTX-I was complete in some but not in all cells. Because resting membrane potential (RMP) of untreated C6 glioma cells was -40.4 ± 1.3 mV (n = 10, Fig. 17B). and was sensitive to DTX-I (-7.9 ± 2.5 mV, n = 10), we suspected that DTX-I sensitive K^+ currents, probably containing Kv1.1 subunits, were responsible for generation of the RMP. Indeed, Northern blot analysis of poly-A^+ RNA prepared from unstimulated and Iso/IBMX stimulated C6 Glioma showed that only Kv1.1 was detectable and not Kv1.2, Kv1.5 or Kv1.6 (data not shown). The depolarizing effect of DTX-I was reversible (after wash out -37.4 ± 3.2 mV, n = 7) (Fig. 17B).

DISCUSSION

Voltage-gated potassium (Kv) channels are involved in determining excitability in a variety of cell types (Hille, 1992). In neurons, Kv channels are likely to be involved in repolarizing the membrane following action potentials and in regulating neurotransmitter release. In lymphocytes, Kv1.3 has been shown to set the RMP which in turn is permissive for proliferation (Leonard et al., 1992). Thus, because of their critical functions, expression as

well as activity of Kv channels must be carefully regulated. We show here that intracellular cAMP levels regulate expression of Kv1.1 by inducing a destabilization of the Kv1.1 transcript.

Previous work has shown that cAMP regulates transcription of Kv1.5, a Kv channel expressed in cardiac and pituitary cells (Levitan et al., 1995; Mori et al., 1995; Mori et al., 1993; Takimoto and Levitan, 1996). Takimoto et al. (1995) have clearly demonstrated that PKA activity is required for basal transcription of Kv1.5 and that Kv1.5 RNA stability is unchanged when PKA activity is inhibited. Promoter elements identified in the 5'-flanking region of the Kv1.5 gene include a CRE (cAMP response element), a GRE (glucocorticoid response element) and a newly identified element KRE (for Kv1.5 Repressor Element). The GRE is likely to be involved in mediating the dexamathsone induced increase in transcription in cells of both pituitary and cardiac origin. Interestingly, the CRE is likely to be involved in the cAMP-stimulated increase in transcription in cells of cardiac origin and decrease in transcription in cells of pituitary origin. These opposing effects on transcription coupled to elevation of cAMP might be explained by the large family of CRE binding proteins, including CREB/CREM, that have been shown to have both stimulatory and inhibitory effects on transcription (de Groot and Sassone, 1993). Analysis of the proximal 1.5kb of promoter region 5' of the Kv1.1 transcriptional start site reveals that it does not contain CRE or

GRE elements and does not respond to cAMP in luciferase reporter gene constructs (See Fig. 5, Chapter 1).

Levin et al. (1995, 1996) reported that elevation of cAMP in *Xenopus* oocytes expressing cRNA encoding the Kv1.1 ORF results in an increase in the levels of Kv1.1 RNA, protein and an increase in amplitude of the resulting delayed-rectifier type K^+ current. The relevance of these experiments to endogenous regulation of Kv1.1 is unclear, however, because only the Kv1.1 ORF - absent any of the Kv1.1 promoter, 5'UTR or 3'UTR regions - was expressed in the amphibian oocyte system. Using a eukaryotic system, Bosma et al. (Bosma et al., 1993) made stable transfections with a construct expressing the Kv1.1 ORF in normal CHO cells and in CHO cells stably transfected with a dominant negative mutation of the PKA regulatory subunit. In contrast to the oocyte system, these studies demonstrated that in cells with a chronic reduction of PKA activity there is a 3-fold increase in Kv1.1 protein and a 3.4-fold increase in whole cell current density. As with the Levin et al. (1995, 1996) studies, the study by Bosma et al. (1993) was done without the Kv1.1 promoter, 5'UTR or 3'UTR region in the Kv1.1 expression constructs. The most likely explanation for the opposing results seen in these studies is the difference in expression systems.

To avoid the limitations of the above studies we investigated the molecular mechanism of regulation of expression of the endogenously

expressed Kv1.1 gene in C6 glioma cells. The results reported here are consistent with the observations of Bosma et al. (1993), namely, that when cAMP levels are elevated Kv1.1 protein is decreased as is the peak current amplitude of a fast-activating, sustained delayed-rectifier type of K^+ current (50% reduction by 12 hours). These decreases were preceded by a 5-fold decrease in the steady state level of Kv1.1 RNA. The rapid reduction in Kv1.1 RNA ($t_{1/2} < 3$ hours) results from post-transcriptional destabilization of the transcript since we can demonstrate at most a 35% reduction in Kv1.1 gene transcription measured at 6 hours. Similar observations have been reported for the β_2-adrenergic receptor (Danner et al., 1998; Danner and Lohse, 1997) and for type 1 angiotensin II receptor (Wang et al., 1997) both of which respond to increased cAMP by decreasing the stability of their RNA without measurable effect on their rates of transcription.

Kv1.1 is expressed strongly in certain neurons in adult rodents (Kues and Wunder, 1992; Veh et al., 1995; Wang et al., 1994). During development, Kv1.1 is expressed in undifferentiated glia in the mouse embryo (Hallows and Tempel, 1998) and in isolated sciatic nerve in early postnatal rats (Chiu et al., 1994). At the electrophysiological level, a sustained outward K^+ current is down regulated during Schwann cell differentiation (Wilson and Chui, 1990). In addition, 24 to 72 hour incubation of cultured oligodendrocytes with TNF-α has been shown to elevate intracellular cAMP levels, leading to a

subsequent down regulation of an outwardly rectifying K^+ current and a decrease in the RMP (Soliven et al., 1991). Our findings show that unstimulated C6 glioma express Kv1.1 and that the resting membrane potential in these cells is determined by a DTX-I sensitive K^+ current. It is possible, therefore, that translational regulation of Kv1.1 expression plays a role in glial proliferation or differentiation.

Why might glia respond to cAMP at the translational level? Given that Kv1.1 has a large genomic unit and a long 5'UTR with predicted extensive secondary structure (see previous chapter), regulating expression of Kv1.1 at the translational level may allow for a more rapid response to changes in cellular cAMP levels. The fact that many other ion channel genes have large untranslated regions suggests that this form of gene regulation - translational regulation - may be an efficient mechanism for regulating cellular excitability.

Table 3. Effect of pharmacological treatments on Kv1.1 mRNA steady-state levels.

	Kv1.1/cyclophilin mRNA level, % of Control			
	2	4	6	24(hours)
KCl_2	---	---	50.2±1.1***	48.9±2.8***
PMA	---	---	41.7±1.6***	43.4±4.0***
A23187	---	---	41.3±3.6***	129.2±9.6***
Forskolin	54.7±0.1*	47.2±3.4*	38.7±1.4*	22.5±0.1***
1,9-dideoxy forskolin	---	---	---	93.6±0.5
8-cpt-cAMP	42.6±1.3*	---	17.5±3.6*	9.4±0.3***
Iso/IBMX	49.7±0.3**	23.8±0.4***	19.8±0.4***	18.5±2.9***
Iso	---	---	47.2±0.7**	---
IBMX	---	---	58.2±0.1*	---

*p≤0.05 **p≤0.005 ***p≤ 0.001

Fig. 12 Kv1.1 mRNA level decreases with elevation of intracellular cAMP.

A: A representative RPA gel showing single samples at 6 and 24 hours for control, forskolin and Iso/IBMX-treated cultures. Undigested, full-length probes (Kv1.1 and cyclophilin) are indicated. Protected bands that were quantitated are boxed in the third lane. The mouse Kv1.1 probe protected two bands in rat-derived C6 glioma. Cyclophilin consistently gave a single band with a smear below. The largest cyclophilin band was used to quantify changes in Kv1.1 mRNA levels. **B:** Kv1.1 RNA levels in C6 Glioma were treated with vehicle (control) or Iso/IBMX for up to 72 hours. Triplicate plates were collected for each treatment group. Cyclophilin levels were unchanged over 12 hours, but declined in both treated and control samples collected at 24, 48 and 72 hours. Kv1.1 levels did not change significantly over 72 hours in control samples. Therefore, the data have been corrected to the cyclophilin levels at 0 hours.

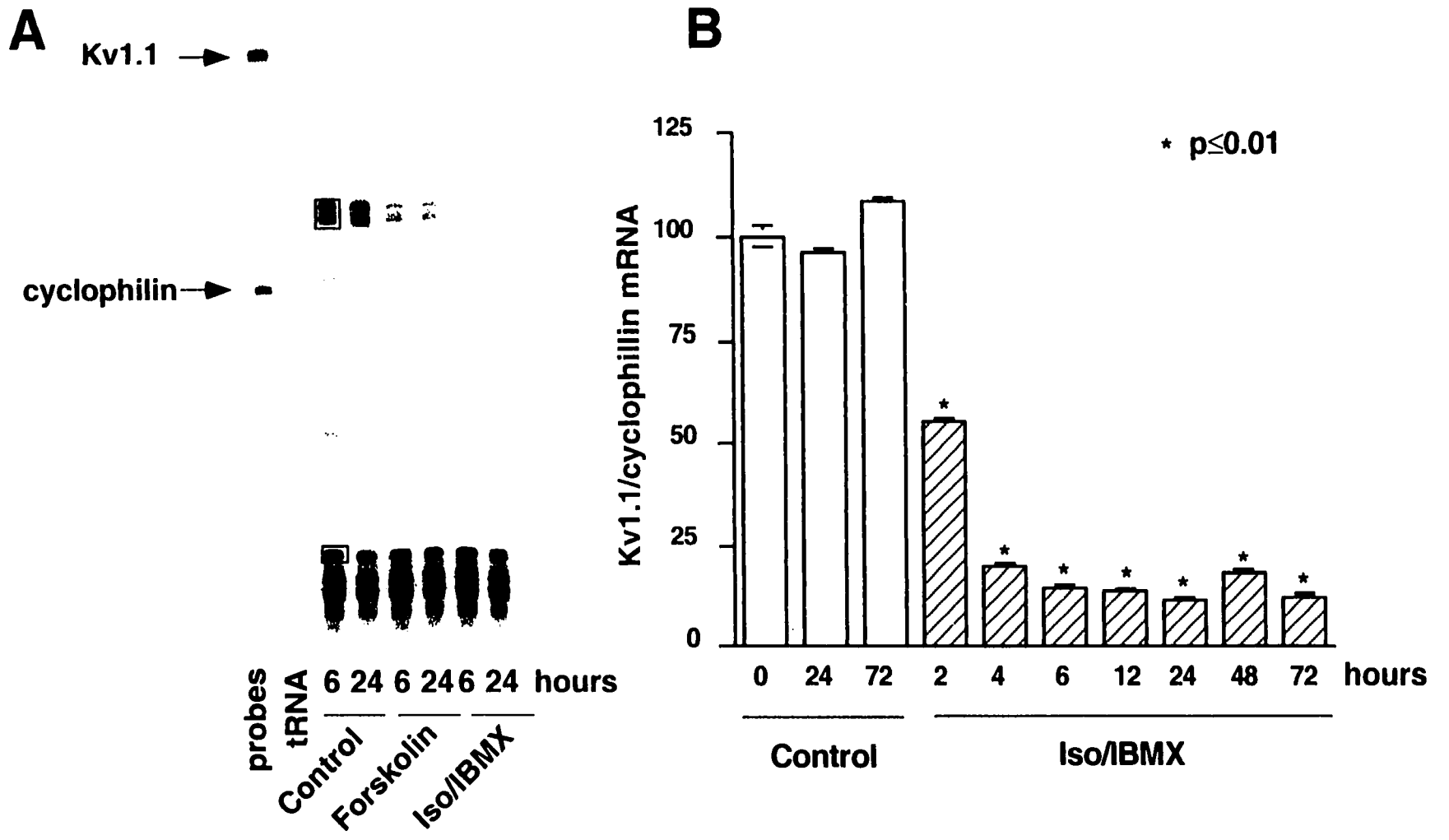

A
Kv1.1
cyclophilin
probes
tRNA
6 24 6 24 6 24 hours
Control
Forskolin
Iso/IBMX
B
* p≤0.01
Kv1.1/cyclophillin mRNA
125
100
75
50
25
0
0 24 72 2 4 6 12 24 48 72 hours
Control
Iso/IBMX

Fig. 13. PKA activity and Kv1.1 mRNA levels are inversely correlated. Paired sets of C6 Glioma cultures were treated with the indicated pharmacological agents for 6 hours then harvested for PKA activity assays (A) and RPA assays (B).

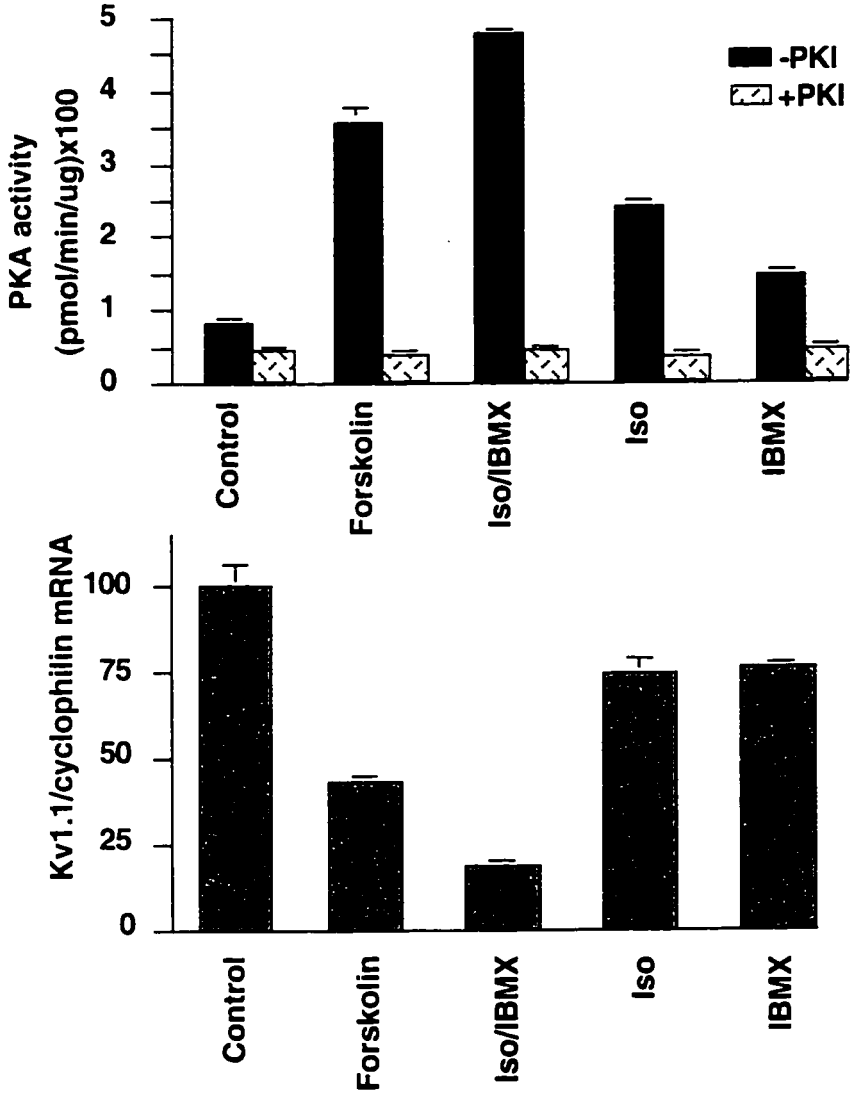

PKA activity
(pmol/min/ug)x100
5
4
3
2
1
0
-PKI
+PKI
Control
Forskolin
Iso/IBMX
Iso
IBMX
Kv1.1/cyclophilin mRNA
100
75
50
25
0
Control
Forskolin
Iso/IBMX
Iso
IBMX

Fig. 14. Elevation of cAMP affects the rate of Kv1.1 RNA degradation but does not affect transcription of Kv1.1. **A:** Kv1.1 RNA half-life was determined by initiating pharmacological treatments and harvesting RNA at the indicated times. RPA analysis was used to follow Kv1.1 RNA levels relative to cyclophilin. Cyclophilin levels did not change significantly with any of these treatments at any of these time points. Half-life measurements were made by plotting results on a semi-log plot. Error bars are not shown since error was ≤ 2% at each time point. **B:** For Nuclear Run-on transcription assays nuclei were prepared from cells treated for 6 hours with vehicle (Control) or Iso/IBMX. Quantitation represents the signal for the antisense strand and is represented as the ratio of Iso/IBMX to Control. On the bottom is a representative radiograph from one of three experiments which showed similar results.

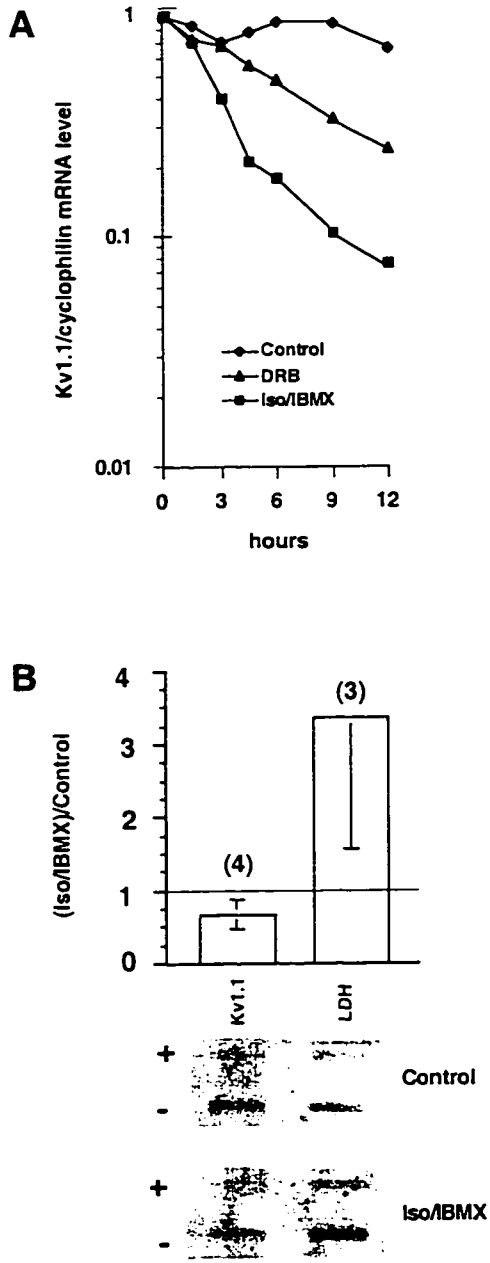

A
1
0.1
0.01
Kv1.1/cyclophilin mRNA level
Control
DRB
Iso/IBMX
0
3
6
9
12
hours
B
4
3
2
1
0
(Iso/IBMX)/Control
(4)
(3)
Kv1.1
LDH
+
-
Control
+
-
Iso/IBMX

Fig. 15 Elevation of cAMP decreases Kv1.1 protein. Cells were treated with vehicle (Control) or Iso/IBMX for the indicated times and a crude membrane fraction was prepared. Western blot analysis was performed with a polyclonal antibody that specifically recognizes Kv1.1 protein. Equal amounts of protein were loaded per lane.

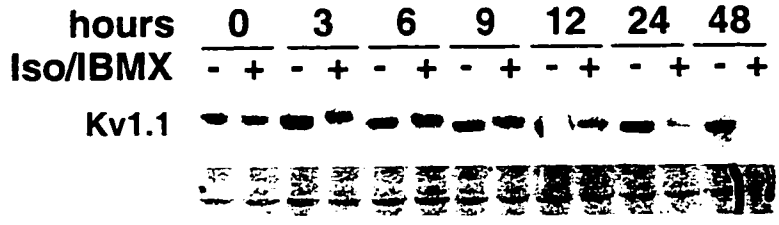

hours
0
3
6
9
12
24
48
Iso/IBMX
- + - + - + - + - + - + - +
Kv1.1

Fig. 16 Elevation of intracellular cAMP level decreases K^+ current density.

A: Representative K^+ currents in a cell treated with vehicle (Control) and in another cell treated with isoproterenol and IBMX for 48 hrs (Iso/IBMX). Currents were elicited by a voltage step from -70 mV to 0 mV in whole-cell configuration. Recordings with typical level of currents were selected for illustration. Membrane capacitance was 20 pF for the control and 15 pF for the treated cell. **B:** Summary of current density in control cells (open bar) or in cells treated with Iso/IBMX (hatched bar) for various incubation times. For group labeled 12 hrs., cells were patch-clamped between 10 and 14 hrs after the start of treatments. Cells were measured between 21 and 27 hrs for group labeled 24 hrs. and between 45 and 51 hrs for group labeled 48 hr. Current density was calculated as current at 0 mV divided by membrane capacitance. Dashed line indicates the current density of control cells at 0 hr. Number of cells measured for each condition is given in parenthesis.

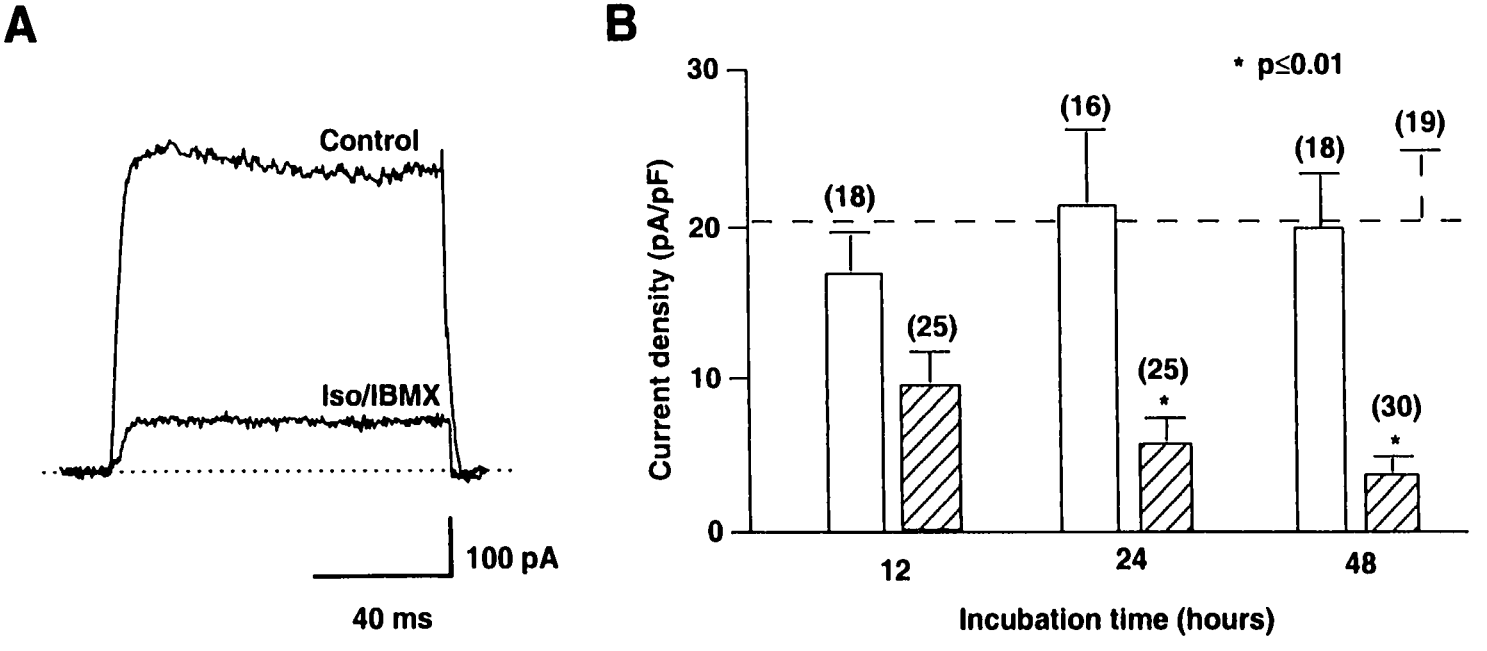
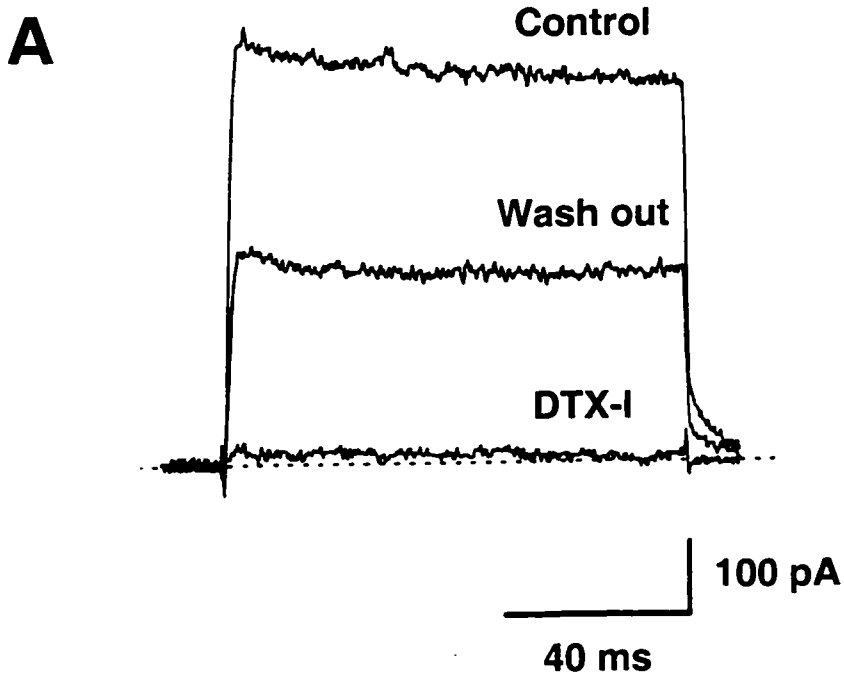
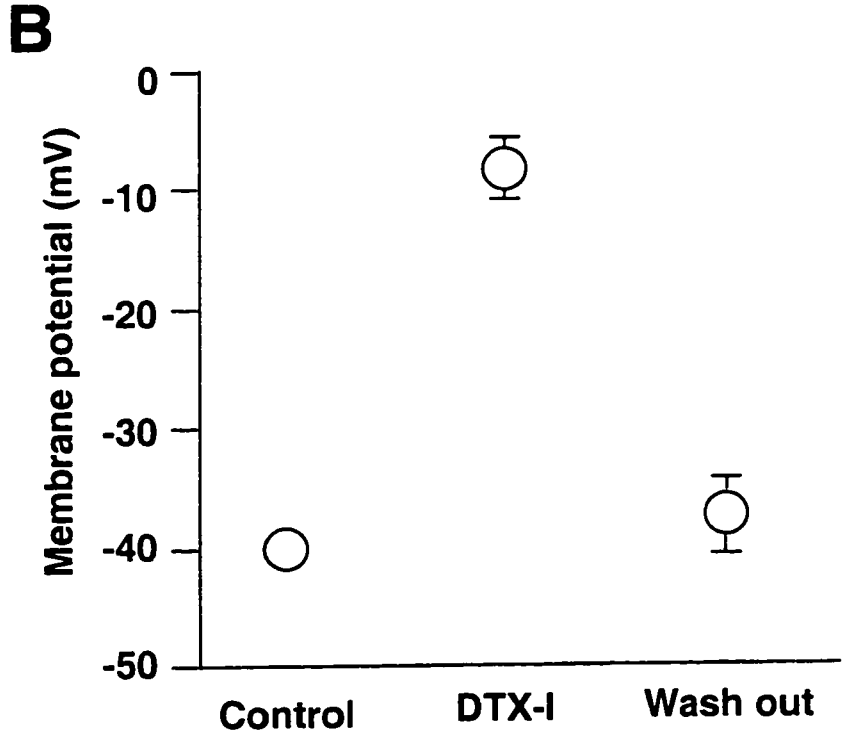

A
Control
Iso/IBMX
100 pA
40 ms
B
* p≤0.01
30
20
10
0
Current density (pA/pF)
(18)
(25)
(16)
(25)
*
(18)
(19)
(30)
*
12
24
48
Incubation time (hours)

Fig. 17 Potassium currents and the resting membrane potential in C6 glioma cells are sensitive to dendrotoxin-I (DTX-I). **A:** Currents were recorded in Ringer's solution (Control), after 2 min of perfusion with 100 nM DTX-I containing Ringer's (DTX-I), and after 3.5 min of wash out with Ringer's (Wash out). K^+ currents were activated by the same voltage protocol as Fig. 6A. **B:** Membrane potential in current-clamp mode (I=0) was measured under the same condition as **A**.


A
Control
Wash out
DTX-I
100 pA
40 ms


B
Membrane potential (mV)
0
-10
-20
-30
-40
-50
Control
DTX-I
Wash out

CHAPTER 4: INVESTIGATION OF SECOND MESSENGER PATHWAY INVOLVEMENT IN DESTABILIZATION OF Kv1.1 mRNA

INTRODUCTION

The results presented in the previous chapter lead one to speculate on what signaling pathway is involved in the observed destabilization of the Kv1.1 transcript. To this end, preliminary data is presented here demonstrating that attempts were made to dissect the signaling pathway by use of various inhibitors. These data are not sufficient alone to present in a journal article and are included here to show that further work on this project was conducted.

METHODS

Reagents:

Tissue culture media, penicillin/streptomycin, and L-glutamine were purchased from GIBCO/BRL. Fetal bovine serum and equine serum were purchased from HyClone. DRB, forskolin, KT5823, H89, Sp-cAMP and Rp-cAMP were purchased from CalBiochem, PD98059 was purchased from Biomol, template for rat-cyclophilin riboprobe from Ambion, radioisotopes from NEN, and recombinant RNasin® inhibitor from Promega. All other reagents were purchased from Sigma.

Cell Culture:

The C6 Glioma cell line was purchased from ATCC and maintained at less-than 80% confluence in F10 medium supplemented with 15% equine serum, 2.5% fetal bovine serum, 2 mM glutamine and 10U/ml penicillin G, 10μg/ml streptomycin sulfate. Cells between passages P41 and P50 were seeded at 2.0 X 10^4 cells/cm^2. Single-dose pharmacological treatments were initiated 24 hours after seeding; cells were harvested at the time periods indicated in the figure legends. For all experiments vehicle was added to untreated control cells.

RNA Analysis:

RNA was extracted from the cells using guanidininum as described (Chomczynski and Sacchi, 1987). For RNase Protection Assay (RPA) 5ug of total RNA per sample was incubated with two ^{32}P-radiolabeled riboprobes; one for mouse Kv1.1 (Bosma et al., 1993) and one for rat cyclophilin. The RPA protocol is that of (Bordonara, 1994) except that Kv1.1 riboprobe was synthesized with 0.4μM UTP in the reaction mixture and cyclophilin with 20μM UTP in the reaction mixture. In the hybridization mixture 5 x 10^5 cpm of Kv1.1 and 1 x 10^5 cpm of cyclophilin were included per sample. The dried gels were exposed to a phosphorimager screen for quantitative analysis. The results are represented as the ratio of Kv1.1 protected fragment/cyclophilin protected fragment. Statistics were calculated using a two-tailed *t* test or a

one-way ANOVA followed by Tukey's multiple comparison analysis as indicated in figure legends.

PDE profile:

Untreated cells were washed twice with PBS, removed from the culture flask, resupended in 1 ml of homogenization buffer (40mM TrisHCl, pH 8.0, 1μg/ml leupeptin, 1μg/ml pepstatin A, 15mM benzamidine) and lysed with 25 strokes of the tight pestle of an ice cold dounce homogenizer. The samples were frozen at -80°C and given to W. Sonnenburg for analysis.

RESULTS

Additional cAMP analogs

To extend the efficacy of elevated cAMP to induce the destabilization of the Kv1.1 transcript, C6 Glioma cells were treated with the various cAMP analogs shown in Fig. 18. The 8-Br-cAMP and db-cAMP are about as effective as Iso or IBMX alone in decreasing the level of Kv1.1 transcript. The Sp-cAMP analog - a relatively specific, non-hydrolizable activator of PKA - caused no reduction in Kv1.1 transcript level. It is possible that the concentration used was too low (see argument below for the entantiomer Rp-cAMP), that this particular batch was inactive or that the reduction in Kv1.1 is not mediated through a strictly PKA signaling pathway.

PKA inhibitors H89 and RP-cAMP

Since the reduction in Kv1.1 transcript seems to be strongly correlated with elevation of intracellular cAMP, inhibitors of PKA activity were used to try to block the reduction in steady state Kv1.1 transcript levels induced by Iso/IBMX treatment. As can be seen from Fig. 19, neither H89 (20μM) nor Rp-cAMP (0.2mM) blocked the decrease in Kv1.1 transcript level induced by Iso/IBMX treatment. These results suggest that either the effect of Iso/IBMX on Kv1.1 transcript stability is not linked to activation of PKA or that neither of these inhibitors were fully able to block the PKA activity in these cells. While the concentration of H89 (20μM) used is typically thought to be sufficient to block PKA, there are apparently some cell lines where this is not true (K. Burton, personal communication). Similarly, the concentration of Rp-cAMP (0.2mM) used is at the highest end published for most cells lines and yet, Papadopoulos & Guarneri (1994) ((Papadopoulos and Guarneri, 1994) and personal communication) indicated in their studies with C6 Glioma that 1.0mM Rp-cAMP was required - fully a 5-fold higher concentration than was attempted in our experiments - to effectively inhibit PKA activity. Therefore, PKA activity assays will need to be done with extracts of cells treated with and without the inhibitors to determine the efficacy of inhibition of PKA.

PDE profile

To determine if there is an unusual phosphodiesterase (PDE) activity in these cells a PDE profile was done by W. Sonnenburg. The results of these assays demonstrates that the major peak of PDE activity was due to PDE1 - a CaM stimulated PDE. The other PDE activities detected, in order of decreasing activity are: PDE2 (cGMP specific-PDE); PDE3 (cGMP inhibited-PDE) and PDE4 (cAMP specific-PDE). This profile does not indicate any unusual pattern of PDE expression.

PKG inhibitor

For several reasons we investigated the role of cGMP dependent protein kinase (PKG) in the Iso/IBMX induced decrease in Kv1.1 transcript. These are: 1) the PDE profile showed that there was a significant amount of PDE2 and PDE3 in the C6 Glioma, 2) that we obtained no inhibition with two specific PKA inhibitors and 3) that Kim et al. (Kim et al., 1995; Kim and Sheng, 1996) have demonstrated an association of guanylate kinases with clustering of Shaker-type K channels. To test the possible role of PKG we treated cells with 8-Br-cAMP, 8-Br-cGMP, Iso/IBMX, the PKG inhibitor KT5823, and the combination of Iso/IBMX/KT5823. As can be seen in Fig. 20, the 8-Br-cAMP (250μM) had no effect on the Kv1.1 transcript level due to the fact that the concentration used was too low since a 4-fold higher concentration (1mM) was effective, (compare to Fig. 18). The 8-Br-cGMP

actually caused a statistically significant increase in the Kv1.1 transcript level comparable to that seen after 24 hours of incubation with the calcium ionophore A23187 (See Table 3, Chapter 3). The inhibitor KT5823 did not block the Iso/IBMX induced decrease in Kv1.1 transcript.

MAP Kinase inhibitor

Another pathway that may be involved in the reduction of the Iso/IBMX induced destabilization of Kv1.1 is that of the MAP Kinase pathway. Kurino et al. (1996) demonstrated that in C6 Glioma and in cortical astrocytes increases in intracellular cAMP levels inhibits both MAP kinase activity and cell proliferation. In breast cancer cells the cAMP analog, 8-Cl-cAMP, was shown to inhibit proliferation and that this was correlated with a reduction in expression of the ras proto-oncogene protein (Strobl et al., 1995). This latter link to the ras signaling pathway is interesting in light of studies in fibroblasts showing K_{Ca} current density was increased through the ras/raf signaling cascade and that this K_{Ca} induction was correlated with increased cell proliferation. (As will be shown in the following chapter we also see a marked inhibition of proliferation of C6 Glioma when treated with Iso/IBMX.) Additionally, there is a set of papers, (Lim and Zaheer, 1996; Zaheer and Lim, 1996; Zaheer and Lim, 1997) that show that PKA phosphorylates Glial Maturation Factor (GMF) which is a specific and potent inhibitor of MAP kinase (ERK1/ERK2) and that the phosphorylated GMF

increases the activity of PKA thereby creating a positive feedback system that will potently downregulate the MAP kinase pathway. Finally, this decrease in the MAP kinase activity is correlated with activation of p38. We used the MAP Kinase inhibitor PD98059 to determine if we could mimic the effect of Iso/IBMX. As can be seen in Fig. 21 we see that the PD98059 inhibitor does cause a statistically significant decrease in Kv1.1 but, it is not of the same magnitude as Iso/IBMX alone. Further, the combination is not additive. Additionally, the more general, non-specific kinase inhibitor staurosporine resulted in a decrease in Kv1.1 of nearly the same magnitude as Iso/IBMX and is likely even additive. Yet, the cells did not appear to be healthy and intact in the presence of this inhibitor - especially at the high concentration (100nM). We conclude that the MAP kinase cascade may play a role in the observed destabilization of Kv1.1 mRNA.

Transcription inhibition with Iso/IBMX treatment

To determine if the Iso/IBMX induced destabilization required on-going transcription we included in the experiments measuring the half-life of Kv1.1 mRNA with the combination of Iso/IBMX + DRB. As can be seen in Fig. 22 the transcription inhibitor prevents the increased rate of destabilization induced by Iso/IBMX. We obtain the same result when we use the transcriptional inhibitor actinomycin D. There is precedence for this in the cAMP-induced post-transcriptional destabilization of the following list of

mRNAs: cilliary neurotropic factor (CNTF) mRNA (Nagao et al., 1995), transcription factor LFB3 mRNA (Marksitzer et al., 1995), β2-adrenergic receptor mRNA (Danner et al., 1998; Danner and Lohse, 1997), type 1 angiotensin II receptor mRNA (Wang et al., 1997), soluble guanylate cyclase (sGC) mRNA (Liu et al., 1997) and clusterin mRNA (Rosemblit et al., 1996). For all but the CNTF mRNA nuclear run-on transcription assays were done to demonstrate that the elevated cAMP levels did not change transcription in either the positive or negative direction. For each of the above mentioned transcripts inhibition of transcription with either DRB or actinomycin D prevented the accelerated destabilization of the mRNA in cells with elevated cAMP levels. The investigators for the transcription factor LBF3 mRNA (Marksitzer et al., 1995), β2-adrenergic receptor mRNA (Danner et al., 1998; Danner and Lohse, 1997), type 1 angiotensin II receptor mRNA (Wang et al., 1997), soluble guanylate cyclase (sGC) mRNA (Liu et al., 1997) and clusterin mRNA (Rosemblit et al., 1996) each postulate that a 'factor' sensitive to transcriptional inhibition was responsible for the cAMP-mediated destabilization. In fact, Danner et al. (1998) have reported initial identification of a cAMP inducible RNA binding protein that binds to the 3'UTR region of the β2-adrenergic receptor mRNA shown to define the *cis*-element instability region (Danner et al., 1998). Based on the similarities on these various

systems, we speculate that some 'factor' is induced by the Iso/IBMX that is involved in the destabilization of the Kv1.1 transcript.

Translation inhibition with CHX

Finally, to try to show that transcription and translation are required for this 'factor' we treated cells with the translation inhibitor, cycloheximide (CHX). As can be seen in Fig. 23 all concentrations of CHX alone result in a decrease in Kv1.1 transcript level. At all concentrations of CHX there is some rescue of the Iso/IBMX induced decrease in Kv1.1 - but not full recovery. This experiment was repeated twice with the same results and are very difficult to interpret. We would need to use another translation inhibitor to attempt to determine if translation is required in the Iso/IBMX mediated destabilization of Kv1.1 RNA. These results suggest perhaps the Iso/IBMX induced destabilization of Kv1.1 is the result of a complex mechanism - perhaps phosphorylation and inactivation of a 'protective' factor on the Kv1.1 transcript as well as transcription-translation of a 'destabilization' factor that causes the degradation of the Kv1.1 transcript.

DISCUSSION

Based on the results with the inhibitors, the most likely link exists between the Iso/IBMX induced destabilization and the MAP kinase signaling pathway. Furthermore, the data presented in this chapter is most consistent with that of (Danner et al., 1998; Danner and Lohse, 1997; Liu et al., 1997;

Marksitzer et al., 1995; Rosemblit et al., 1996; Wang et al., 1997) where there is likely a 'factor' that is under transcriptional control that is implicated in the Iso/IBMX induced destabilization of Kv1.1 mRNA.

Fig. 18. Analysis of various cAMP analogs on reduction of steady state Kv1.1 mRNA levels. Cells were treated for 6 hours with each of the indicated cAMP analogs. 8-Br-cAMP and db-cAMP were each used at 1mM; 8-cpt-cAMP and Sp-cAMP were each used at 100μM; Iso was used at 10μM and IBMX was used at 500μM. RNA was extracted from the cells and RPA analysis was conducted measuring Kv1.1 relative to cyclophilin. For each treatment n=3. Error bars represent S.D. Statistical analysis was done with a two-tailed Student's *t*-test.

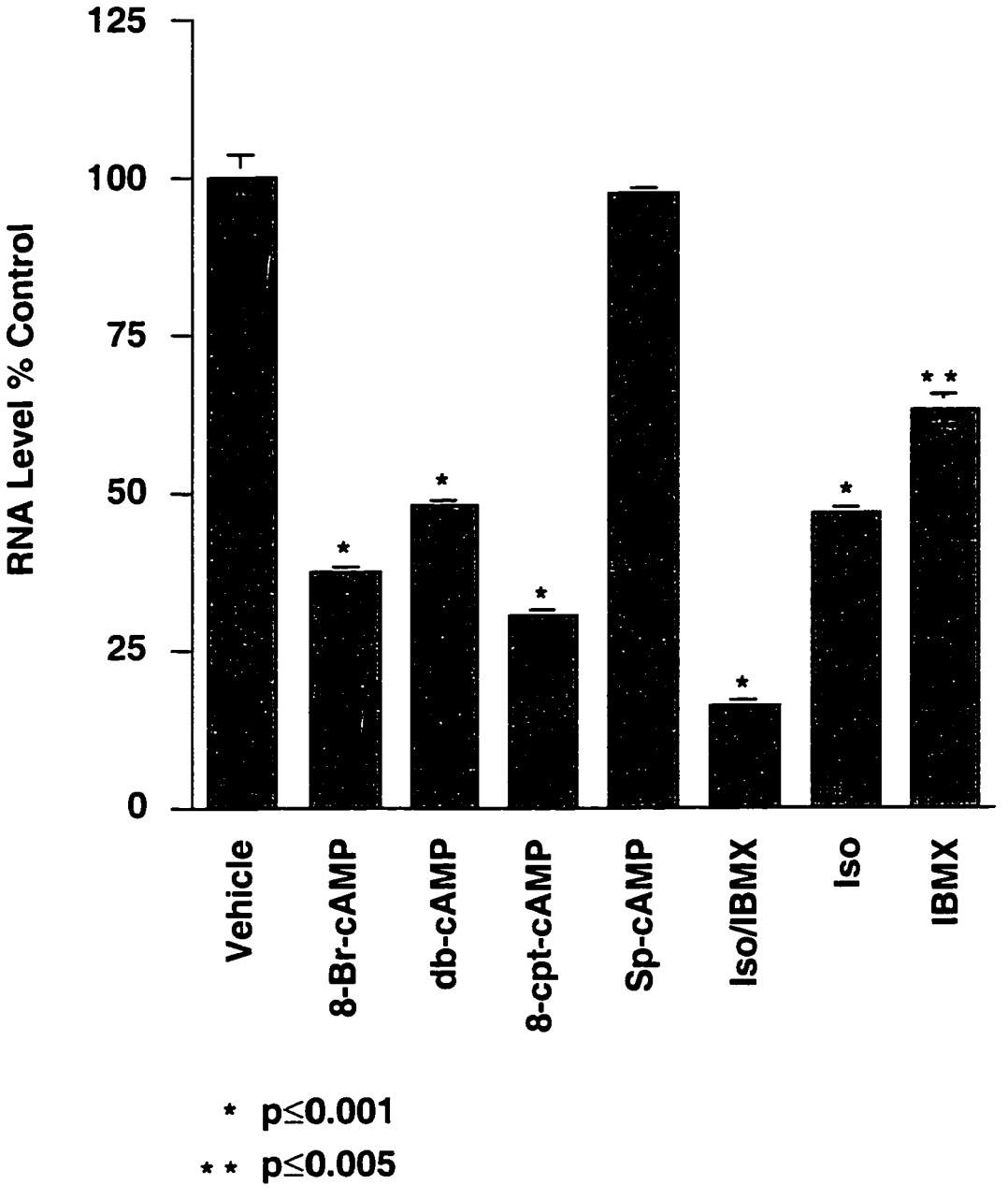

RNA Level % Control
125
100
75
50
25
0
Vehicle
8-Br-cAMP
db-cAMP
8-cpt-cAMP
Sp-cAMP
Iso/IBMX
Iso
IBMX
* p≤0.001
** p≤0.005

Fig.19. Analysis of inhibitors of PKA on reduction of steady state Kv1.1 mRNA levels. Cells were treated for 6 hours with each of the indicated treatments. Rp-cAMP was used at 0.2mM, Iso at 10μM, IBMX at 500μM, and H89 at 20μM. RNA was extracted from the cells and RPA analysis was conducted measuring Kv1.1 relative to cyclophilin. For each treatment n=3. Error bars represent S.D. Statistical analysis was done with a two-tailed Student's *t*-test.

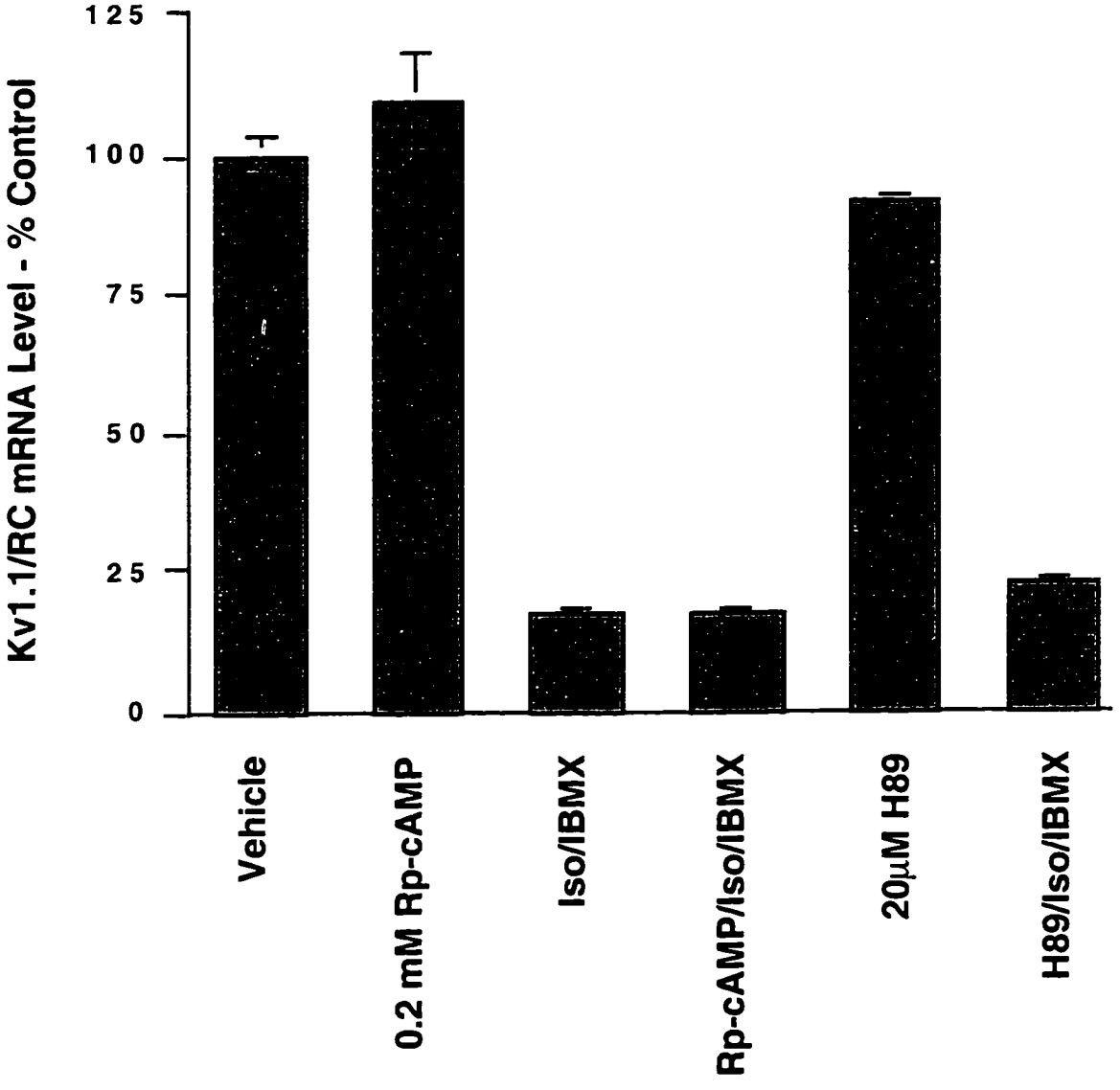
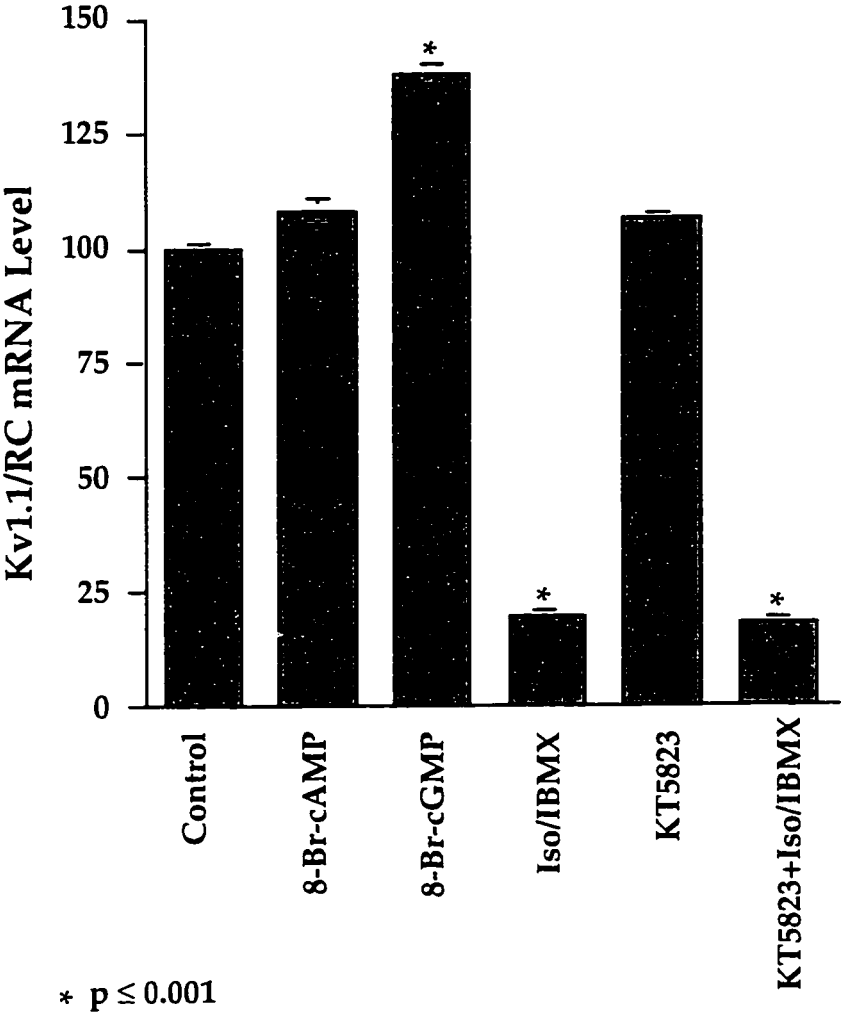

Kv1.1/RC mRNA Level - % Control
125
100
75
50
25
0
Vehicle
0.2 mM Rp-cAMP
Iso/IBMX
Rp-cAMP/Iso/IBMX
20μM H89
H89/Iso/IBMX

Fig. 20. Analysis of cGMP and cGMP Kinase on reduction of steady state Kv1.1 mRNA levels. Cells were treated for 6 hours with each of the indicated treatments. 8-Br-cAMP and 8-Br-cGMP were each used at 250μM, Iso at 10μM, IBMX at 500μM, and KT5823 at 1μM. RNA was extracted from the cells and RPA analysis was conducted measuring Kv1.1 relative to cyclophilin. For each treatment n=3. Error bars represent S.D. Statistical analysis was done with a two-tailed Student's *t*-test.


Kv1.1/RC mRNA Level
150
125
100
75
50
25
0
*
*
*
Control
8-Br-cAMP
8-Br-cGMP
Iso/IBMX
KT5823
KT5823+Iso/IBMX

* $p \leq 0.001$

Fig. 21. Analysis of MAP Kinase inhibitors on reduction of steady state Kv1.1 mRNA levels. Cells were treated for 6 hours with each of the indicated treatments. PD98059 was used at 100μM, Iso at 10μM, IBMX at 500μM and Staurosporine as indicated. RNA was extracted from the cells and RPA analysis was conducted measuring Kv1.1 relative to cyclophilin. For each treatment n=3. Error bars represent S.D. Statistical analysis was done with a two-tailed Student's *t*-test.

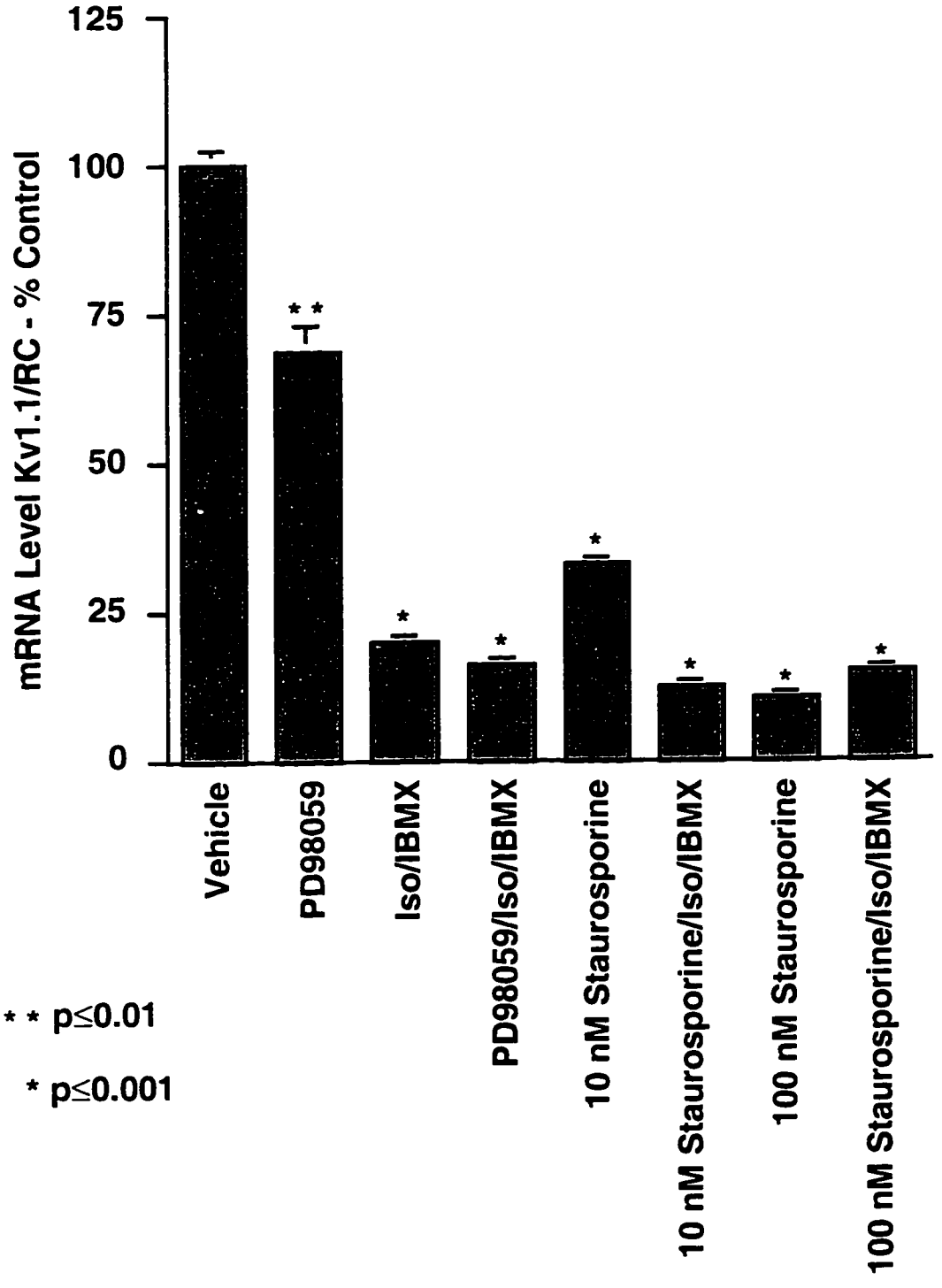

mRNA Level Kv1.1/RC - % Control
125
100
75
50
25
0
Vehicle
PD98059
Iso/IBMX
PD98059/Iso/IBMX
10 nM Staurosporine
10 nM Staurosporine/Iso/IBMX
100 nM Staurosporine
100 nM Staurosporine/Iso/IBMX
* * p≤0.01
* p≤0.001

Fig. 22. Transcription inhibition rescues the accelerated degradation of Kv1.1 RNA. Kv1.1 RNA half-life was determined by initiating pharmacological treatments and harvesting RNA at the indicated times. RPA analysis was used to follow Kv1.1 RNA levels relative to cyclophilin. Cyclophilin levels did not change significantly with any of these treatments at any of these time points. Half-life measurements were made by plotting results on a semi-log plot. Error bars are not shown since error was ≤ 2% at each time point.

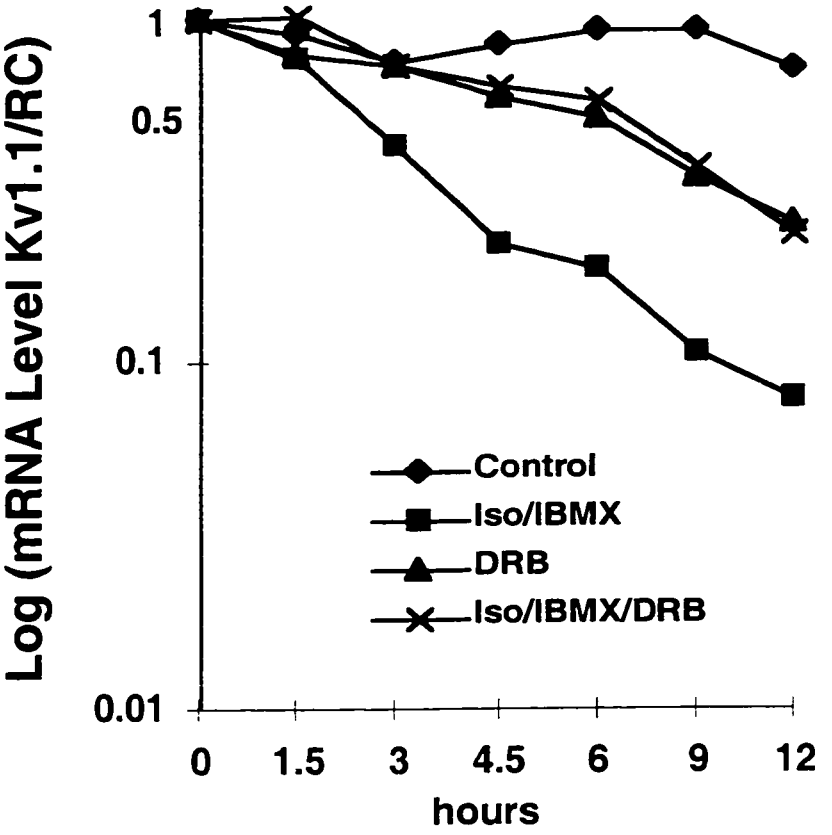
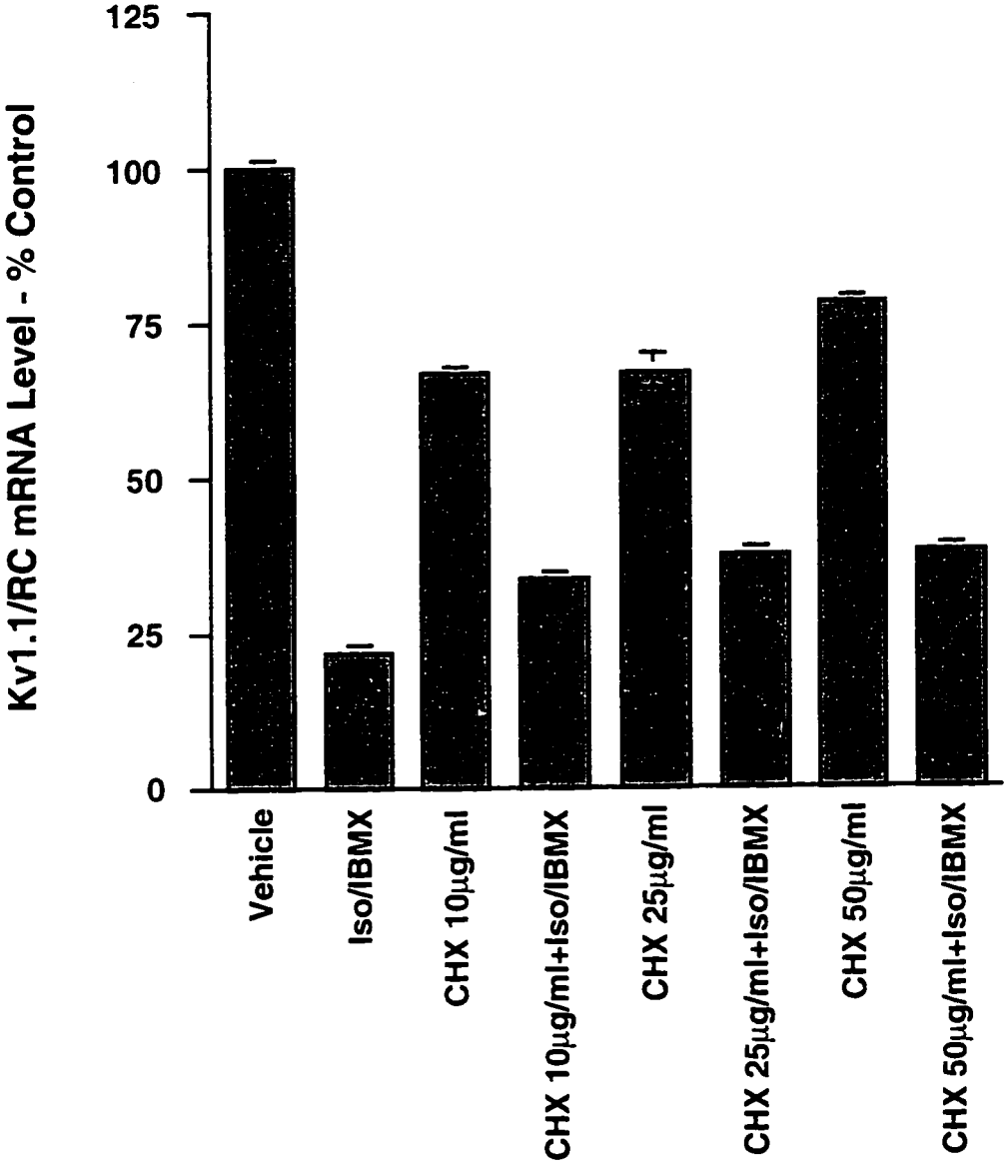

Log (mRNA Level Kv1.1/RC)
1
0.5
0.1
0.01
0
1.5
3
4.5
6
9
12
hours
Control
Iso/IBMX
DRB
Iso/IBMX/DRB

Fig. 23. Analysis of translation inhibition on the Iso/IBMX induced accelerated degradation of Kv1.1 RNA. . Cells were treated for 6 hours with Iso/IBMX and cycloheximide (CHX) in the combinations indicated. Iso was used at 10μM, IBMX was used at 500μM, and CHX at the indicated concentrations. RNA was extracted from the cells and RPA analysis was conducted measuring Kv1.1 relative to cyclophilin. For each treatment n=3. Error bars represent S.D.


Kv1.1/RC mRNA Level - % Control
125
100
75
50
25
0
Vehicle
Iso/IBMX
CHX 10μg/ml
CHX 10μg/ml+Iso/IBMX
CHX 25μg/ml
CHX 25μg/ml+Iso/IBMX
CHX 50μg/ml
CHX 50μg/ml+Iso/IBMX

CHAPTER 5: EFFECT OF POTASSIUM CHANNEL INHIBITORS ON PROLIFERATION OF C6 GLIOMA

INTRODUCTION

The correlation of potassium channels in setting the resting membrane potential (RMP) and proliferation has been studied in a number of cell types including brown fat cells, breast cancer cells, melanocytes, lymphocytes, oligodendrocytes, astrocytes and Schwann cells. Pappone and Oritz (1993) demonstrated that blockers of K channels (TEA, quinine and verapmil) inhibited proliferation of brown fat cells. This effect was not seen with blockers of $Kv_{,Ca}$ channels (apamin, TEA). In a previous study (Pappone and Lucero, 1992) these authors demonstrated that these K channel inhibitors did not prevent hormonally induced heat production so they concluded that the block to proliferation was not due to metabolic changes.

In human melanoma cells Nilius et al. (1990) detected a high K channel density in proliferating cells and almost no detectable K channels in differentiated cells. Further studies with these melanoma cells (Nilius and Wohlrab, 1992) demonstrated that TEA and acute applications of membrane permeant analogs of cAMP (8-cpt-cAMP) blocked this K^+ current. Furthermore, continuous culture with either the K channel blocker, TEA, or

with 8-cpt-cAMP inhibited proliferation of the melanoma cells. Woodfork et al. (1995) found that inhibition of K_{ATP} channels in human breast cancer cells inhibited proliferation and that the cells arrest in the G0/G1 phase of the cell cycle. While the K_{ATP} channels were shown to be required for the G1/S transition, Strobl et al. (1995) postulated that K_{Ca} channels are required for the accelerated progression through S-phase of the cell cycle in these breast cancer cells. Another interesting observation in the breast cancer cells was that the cAMP analog, 8-Cl-cAMP, inhibited proliferation and that this was correlated with a reduction in the ras proto-oncogene protein expression (Strobl et al., 1995) . This latter link to the ras signaling pathway is interesting in light of studies in fibroblasts showing K_{Ca} current density was increased through the ras/raf signaling cascade and that this K_{Ca} induction was correlated with increased cell proliferation. This induced proliferation was blocked by the K channel blocker, TEA (Huang and Rane, 1994).

The role of K channels in setting the RPM and regulating proliferation has been most extensively studied in lymphocytes. In one of the first reports to demonstrate that T-lymphocytes contain Kv channels, Matteson & Deutsch (1984) determined that lymphocytes which had been stimulated with mitogen for 20 hours had a 1.9-fold increase in the observed K^+ current. In a companion paper DeCoursey et al. (1984) also demonstrated that T-lymphocytes contained Kv channels. These authors demonstrated that

mitogen stimulation shifted the gating kinetics of the channel such that the channels opened more rapidly and at more negative membrane potentials. These authors also demonstrated that several K channel blockers effectively inhibited mitogen stimulated proliferation without affecting cell viability. Lee et al. (1986) clearly demonstrated that there was an increase in K channel density in mitogen stimulated proliferating cells. The timing of this increase correlated with the G1/S transition phase of the cell cycle. The K channel blocker, quinine prevented the mitogen induced proliferation and the quinine treated stimulated cells accumulated in the G1 phase of the cell cycle. Using flow cytometry and detection of cell-surface markers expressed at specific cell cycle stages Amigorena et al. (1990) determined that potassium channel blockers inhibited B-cell proliferation at a midpoint of G1. In myeloblastic leukemia ML-1 cells potassium channel blockade was shown to correlate with dephosphorylation of retinoblastoma protein (pRB) and an inhibition of cell-cycle progression through G1/S transition (Wang et al. 1996). A lymphocyte K channel was cloned and identified as Kv1.3 by Douglass et al. (1990). The use of toxins that block K channels - charybdotoxin (ChTX), and more specifically for Kv1.3, noxiustoxin (NxTX) and margatoxin (MgTX) has demonstrated that Kv1.3 controls the RMP in lymphocytes. The toxins prevent depolarization coupled increase in $[Ca^{2+}]_i$ and the consequence is block of the calcium-dependent pathways leading to proliferation

(Kaczorowski and Koo, 1994; Leonard et al., 1992; Lin et al., 1993). Interestingly, Freedman et al. (1995) demonstrate for the first time that Kv1.1 is found in immature $CD4^-CD8^-$ thymocytes and that when this channel is blocked by the specific toxin, DTX, proliferation is inhibited but viability is unaffected.

There have been many reports that K channel expression is correlated with proliferation and differentiation in Schwann cells, oligodendrocytes and astrocytes. In explant cultures of rabbit sciatic nerves, Chiu & Wilson (1989) demonstrated that there was an upregulation, principally of a delayed-rectifier outward K^+ current detected electrophysiologically that paralleled the proliferation of the Schwann cells. The increase in the I_K was accompanied by a hyperpolarization of the cell RMP. The K channel blockers TEA, 4AP and quinine were able to reversibly block the proliferation in a concentration dependent manner at concentrations that blocked I_K. Proliferation was also blocked when the culture medium was changed such that the cells would be depolarized (high $[K^+]_o$, $[Rb^+]_o$, $[Cs^+]_o$ and $[NH_4^+]_o$), a condition that mimics the effects of the K channel blockers on RMP. In a similar study Shrager & Novakovic (1995) demonstrated with explants of mouse spinal cord that TEA or depolarizing culture conditions (high $[K^+]_o$) prevented normal myelination. In this study the sodium channel blocker, tetrodotoxin (TTX) had no effect on myelin development. Consistent with the explant data, in

cultured dissociated Schwann cells there was an increase in outwardly gated $I_{K,o}$ current during proliferation and K channel blockers inhibited proliferation which was correlated with a depolarization of the RMP (Chiu, 1991; Konishi, 1989; Wilson and Chui, 1993; Wilson and Chui, 1990). In fact Wilson & Chiu (1990) show a strong correlation with the decrease in proliferation, the decrease in $I_{K,o}$ and the onset of myelination (Wilson and Chui, 1990), Fig. 5 and Fig. 10).

The regulation of myelination has been shown to be a complex process that requires, in part, axon-Schwann cell contact. When studied in *in vitro* systems many growth factors have been shown to influence onset of myelin formation (Bray et al., 1981; Davis and Goodearl, 1991; Sobue et al., 1983; Sobue et al., 1986; Wilson and Chui, 1993). Consistent with the many studies cited which use ICC, histology, and/or cell morphology to monitor the regulation of myelin formation, other researchers have shown that upon stimulation with axolemmal fractions or growth factors there are changes in the transcriptional regulation of many of the myelin associated gene products, such as P_0, MBP (myelin basic protein), and GalC (galactocerebroside) (Lemke and Chao, 1988; Lemke et al., 1991; Lemke et al., 1990; Zorick and Lemke, 1996). Elevation of intracellular cAMP levels has been demonstrated to mimic the effect of axon contact on induction of myelin formation in cultured Schwann cells (Jessen and Mirsky, 1991; Lemke and Chao, 1988; Raff

et al., 1978; Raff et al., 1978; Sobue et al., 1986). Additionally, elevation of cAMP has been shown to modulate the effect of growth factors on both proliferation and differentiation of cultured Schwann cells and the effects are dependent on the concentration of cAMP, where low concentrations were shown to be mitogenic but high concentrations were correlated with onset of myelination (Sobue et al., 1986). There are also specific growth factor-cAMP combinations that are either mitogenic or have no effect, specifically glial growth factor (GGF) in combinations with low concentrations of cAMP is mitogenic (Davis and Goodearl, 1991; Sobue et al., 1983; Wilson and Chui, 1993) but axolemmal fractions plus elevated cAMP is only mitogenic in combination with other growth factors (bFGF, aFGF and PDGF) (Davis and Goodearl, 1991; Raff et al., 1978; Sobue et al., 1983). A further caveat that must be considered when interpreting these varied studies is that Jessen and Mirsky (1991) have demonstrated that whether cAMP induces proliferation or myelinogenic differentiation of cultured Schwann cells depends on the culture conditions. Specifically, if the culture conditions support proliferation where the medium contains high serum or growth factors (PDFG, FGF etc.) then cAMP is mitogenic. However, if the cells are in a quiescent state - low serum or high density - then cAMP induces differentiation. How these factors play out in vivo is not yet clear but it has been demonstrated that Schwann cells migrate from the embryonic origin in

the neural crest out to the PNS, undergo a period of rapid proliferation followed by differentiation (Bray et al., 1981; Lemke, 1992; Mirsky and Jessen, 1996; Morell, 1984). At each of these points of development the Schwann cells will encounter differing milieu of growth factors and axolemmal contacts - each influencing the pattern and progression of myelination (Mirsky and Jessen, 1996; Zorick and Lemke, 1996).

Many investigators, using many techniques have demonstrated that there is a similar pattern of changes in the expression of K channels during the proliferation and differentiation of glia of the CNS as that seen for Schwann cells of the PNS and these studies have been reviewed extensively (Barres, 1991; Barres, 1991; Kettenmann et al., 1991; Raff and Lillien, 1988; Ransom and Sontheimer, 1995; Sontheimer, 1994; Sontheimer et al., 1989). In the CNS there are a minimum of two types of progenitor cells one giving rise to type 1 astrocytes and the other giving rise to bipotential O2-A cells which give rise to type 2 astrocytes and oligodendrocytes with the latter giving rise to the myelinating cell of the CNS. Essentially it was demonstrated that proliferating O2-A progenitors had relatively high levels of $I_{K,O}$ and no or barely detectable levels of I_{Kir}. As the progenitors differentiated there was a shutdown of the $I_{K,O}$ and a concomitant increase in I_{Kir}. For a schematic diagram that correlates the switch in ion channel expression with the differentiation progression of the glial cells see Kettenmann et al. (1991, Fig. 7)

In the few studies where RMP was measured, the switch from $I_{K,O}$ to I_{Kir} was correlated with a hyperpolarization of the RMP (Pappas et al., 1994; Roy and Sontheimer, 1995; Soliven et al., 1991; Sontheimer, 1994). As with previous cell types, K channel blockers were shown to block proliferation of the oligodendrocyte progenitors (Attali et al., 1997; Pappas et al., 1994; Sontheimer, 1994). It has been postulated that this is related to the fact that at least one consequence of K channel blockers is a change in the RMP (Sontheimer, 1994). Though, Pappas et al. (1994) attribute the K channel blockers effect on inhibiting proliferation to an alkaline shift in the pHi. As with Schwann cells, glial-axon cell contact induces differentiation (Barres, 1991; Lillien and Raff, 1990; Raff et al., 1985; Raff and Lillien, 1988) and elevation of cAMP can mimic the neuronal induced differentiation - namely morphological changes and expression of cells surface markers. The role of cyclic nucleotides in cultures of astroglial cells has been reviewed by Hamprecht (1986). The effect of acutely increasing cAMP concentrations - using either β-adrenergic receptor agonists or with membrane permeable analogs of cAMP results in a decrease in $I_{K,D}$ and a commitment hyperpolarization of the RMP (Hosli et al., 1982; Soliven et al., 1988). When cultured astroglia were incubated with TNF-α for periods of 24 to 72 hours, cAMP levels increased, the morphology changed, the RMP hyperpolarized

and $I_{K,D}$ was reduced (Soliven et al., 1991). In contrast to these examples of the effect of cAMP on RMP, McKhann et al. (1997) found that although cAMP induced a morphological change in cultured astrocytes, there was no correlation to RMP or time in culture (i.e. age/differentiation stage of the cell). Finally, two groups have demonstrated that signaling through AMPA receptors blocks Kv channels in oligodendrocyte progenitor cells (Borges and Kettenmann, 1995; Gallo et al., 1996; Knutson et al., 1997). The result of chronic AMPA mediated block is a decrease in $I_{K,D}$, an increase in I_{Kir}, a hyperpolarization in the RMP, a block to proliferation but not a block in migration of these oligodendrocyte progenitor cells (Gallo et al., 1996; Knutson et al., 1997). This latter fact fits well with the data & hypothesis of Temple and Raff (1986) that astrocytes are predetermined to have only a limited number of divisions prior to undergoing differentiation. Since astrocytes migrate from the original progenitor position, it is interesting to speculate that the cells may tightly regulate the K channel expression in order to suppress proliferation (advancement of the clock) during the migration phase, then up-regulate the channels when arriving at the final position where they proliferate prior to differentiation and down regulation of the channels. Thus, it appears that many of the mechanisms that regulate proliferation and differentiation are the same in the Schwann cells of the PNS and the astrocytes and oligodendrocytes of the CNS.

C6 Glioma cells were established from a glial tumor induced in rat with N-nitrosomethylurea (Benda et al., 1968) and have been extensively used as a model system as astrocyte progenitor cells (Mangoura et al., 1989; Mares et al., 1981; Pfeiffer et al., 1977). At low passage numbers these cells respond to elevated cAMP levels with characteristic morphology change and an inhibition to proliferation (Antonow et al., 1984; Gubits et al., 1992; Messens and Slegers, 1992; Sharma and Raj, 1987; Zorn et al., 1993). At high passage number the C6 Glioma cells were shown to have a greatly diminished capacity to elevate cAMP in response to β-adrenergic receptor agonist stimulation (Gubits et al., 1992). C6 Glioma in which cAMP levels were elevated with db-cAMP were shown to have disrupted passage through the G2 phase of the cell cycle and cells which did pass through G2 accumulated in the G0/G1 phase of the cell cycle (Mares et al., 1991). Electrophysicological studies on C6 Glioma identified Kv1.1 as likely the major K channel responsible for $I_{K,O}$ currents (Wang et al., 1992). Other K channels identified in C6 Glioma cells are small conductance $I_{K,Ca}$ (Manor and Moran, 1994) and a Ca^{2+} and pH dependent K channel (Strupp et al., 1993). Thus, because of the many examples where K channels have been demonstrated to be involved in proliferation and that elevated cAMP in C6 Glioma inhibits proliferation combined with our data (chapter 3) that elevated cAMP in C6 Glioma decreases Kv1.1 expression, we tested whether or not inhibitors of K channels

would block proliferation of C6 Glioma. Our data show that elevation of cAMP does block proliferation. The K channel blocker 4AP was as effective an inhibitor of proliferation as elevated cAMP, in contrast, TEA inhibited proliferation but with a slight delay as compared to 4AP. In our experimental system, the specific Kv1.1 potassium channel toxin, DTX-I, was ineffective at blocking proliferation (though this negative result may be due to degradation of the toxin in the culture medium). These results suggest that functional K channels in general, and possibly Kv1.1 specifically, are permissive for proliferation of C6 glioma.

METHODS

Reagents:

Tissue culture media, penicillin/streptomycin, and L-glutamine were purchased from GIBCO/BRL. Fetal bovine serum and equine serum were purchased from HyClone, radioisotope from NEN. TEA was purchased from Kodak Chemical Co. All other reagents were purchased from Sigma. Dendrotoxin-I (DTX-I) was either a gift from D. Owen or a partially purified preparation made by T. Martin.

Cell Culture:

The C6 Glioma cell line was purchased from ATCC and maintained at less-than 80% confluence in F10 medium supplemented with 15% equine serum,

2.5% fetal bovine serum, 2 mM glutamine and 10U/ml penicillin G, 10µg/ml streptomycin sulfate. Unless otherwise stated cells between passages P41 and P50 were seeded in 12 well cluster dish in 2.5 ml medium. Pharmacological treatments were initiated 24 hours after seeding the culture medium was removed, the cells washed twice with F10 medium and 2.5ml of F10 medium with 1% BSA (fraction V, w/v), 2mM glutamine, 10U/ml penicillin G and 10µg/ml streptomycin sulfate. Treatments were set up in triplicate or quadruplet wells; cells were counted and/or treated with ^{3}H-Thymidine and harvested at the time periods indicated in the figure legends. For all experiments vehicle was added to untreated control cells.

Cell counts

For cell counts a reticule (20mm) with a 4 x 4 grid was inserted into the eye-piece (10X magnification) of an Olympus CK2 inverted microscope with a 10X plan objective. Two fields of cells per well in each of the triplicate wells were counted at 24 hour intervals over the time course of the experiments.

3H-Thymidine incorporation

For ^{3}H-Thymidine incorporation 1µCi of isotope (NEN methyl-^{3}H-Thymidine, 70 -90 Ci/mmol) in 50 µl of medium was added to each well of 12 well cluster dish and the dish was gently but thoroughly mixed. The plate was further incubated for 2 hours at which time the medium was aspirated,

the wells washed twice with ice cold PBS and 0.5 ml of 5% TCA was added. The plate was frozen at -20°C. After all the plates had been collected, the plates were thawed on ice, the wells individually scrapped with a rubber policeman and the contents transferred to a 10 x 75 glass tube, the wells washed with 1 ml of ice cold 5% TCA and the wash combined with the contents of the glass tube. The tubes were centrifuged at 5,000x*g* for 5 minutes at 4°C. The supernatant was aspirated and 0.5ml of 1N NaOH was added. Once the pellets were dissolved in the NaOH, 100µl was counted in a scintillation counter.

Photography

Photography was done on an inverted microscope with a Nikon camera. Film was Kodak TMAX400. Exposures were timed using the light meter of the camera and one stop on either side of the optimal setting (0.25, 0.5 and 1.0 seconds).

RESULTS

Many investigators have documented that elevated cAMP levels inhibit proliferation and induces morphological changes in C6 Glioma. As shown in the previous chapter, we have used Iso/IBMX to elevate the cAMP level in C6 glioma. Furthermore, we clearly demonstrated a strong inverse correlation with cAMP elevation and down regulation of expression of the K

channel, Kv1.1. Here we detail experiments that we have done to determine the effect on proliferation of Iso/IBMX and also the K channel blockers 4AP, TEA and DTX-I.

To document the morphological change induced by the treatments photographs were taken at 75 hours of chronic treatment. As can be seen in Fig. 24 Iso/IBMX causes a marked retraction of the cytoplasm and the cells take on a more spindly appearance. The cells treated with 4AP also have a somewhat more retracted cytoplasm compared to control (Fig. 24). For both Iso/IBMX and 4AP treatments it is clear from the photographs that there are far fewer cells compared to control cells. The cells treated with TEA have undergone no morphological changes compared to control cells (Fig. 24).

Using cell counts as one proliferation assay, we determined that Iso/IBMX and 4AP are nearly equal in their ability to inhibit proliferation (Fig. 25) completely. Interestingly, the inhibition of proliferation with TEA was not significantly different from control treated cells until 48 hours of continuous incubation (Fig. 25).

To confirm this inhibition of proliferation data with a more sensitive assay of proliferation, we did a 2 hour pulse with ^{3}H-Thymidine and measured incorporation on each day of treatment. As can be seen in Fig. 26 we obtained nearly identical results with ^{3}H-Thymidine incorporation as that obtained with cell counts - namely that Iso/IBMX or 4AP effectively stopped

proliferation and TEA inhibited proliferation, but with a delay of at least 48 hours. In this assay, we also tested the effect of the relatively specific Kv1.1 channel blocker, DTX-I (Fig. 26). Fresh DTX-I was added every 48 hours throughout this experiment and aliquots of the 'spent' supernatant were saved and tested electrophysiologically to determine if the toxin was still active. As can be seen in Fig. 26, DTX-I did not inhibit proliferation of the C6 Glioma cells. The results of the electrophysiology showed that the toxin was still active but, was less able to block the $I_{K,O}$ than toxin that had not been in culture for 48 hours.

DISCUSSION

A strong correlation has been made between proliferation and expression of K channels for many different cell types where the higher expression of Kv channel is correlated with proliferation. In a few instances there has been a positive correlation with Kv channel and an inverse correlation with Kir channel expression and proliferation. Additionally, for Schwann cells of the PNS and oligodendrocyte/astrocyte progenitors of the CNS elevation of cAMP levels is one cue that can induce the cells to undergo differentiation into the myelinating phenotype.

Recent studies have been done to identify the specific K channel sub-types that are present in Schwann cells, their sub-cellular localization and

expression pattern during different stages of differentiation. Electrophysiology and pharmacology were used to tentatively identify as Kv1.1 one of the $I_{K,O}$ currents detected in cultured Schwann cells obtained from rabbit sciatic nerve (Baker and Ritchie, 1996). The transcripts for Kv1.1 and Kv1.2 were found in RNA prepared from acutely dissociated sciatic nerve but not from cultured Schwann cells. Furthermore, the levels of Kv1.1 and Kv1.2 mRNA gradually increased between P0 and P15 and then declined to low or undetectable levels at P90 (Chiu et al., 1994). Hallows and Tempel (1998) used ICC to show that Kv1.1 is highly expressed in the putative Schwann cell progenitors within the neural crest of mouse embryos. Then, consistent with Chiu et al. (1994) and Wang et al. (1995), Kv1.1 is at a very low level in Schwann cells of postnatal and adult mice. Immunocytochemistry (ICC) was used to demonstrate Kv1.1 and Kv1.2 localization in the axolemmal compartment at juxtaparanodal regions of the nodes of Ranvier of myelinated axons (Rasband et al., 1998; Wang et al., 1995; Wang et al., 1993). Several methods, ICC, RPA and in situ hybridization, were used to show that expression of Kv1.1 was upregulated in glia and axons of the dysmyelinating mouse mutant *shiverer* (Wang et al., 1995). Using ICC Mi et al. (1995) and Rasband et al. (1998) have demonstrated that Kv1.1 is found in Schwann cells but in fully differentiated cells it could only be detected very faintly in a peri-nuclear compartment whereas in Schwann cells associated with

remyelinating axons Kv1.1 was localized diffusely throughout the cytoplasm. In contrast, Kv1.5 was found in the Schwann cell membrane at the nodes of Ranvier and in bands running along the myelin sheath. Thus it is interesting to speculate that Kv1.1 (and possibly Kv1.2) is expressed in Schwann cells during periods of proliferation and that the expression is down-regulated at a time that is correlated with differentiation. Is the cAMP signaling that is seen with the onset of myelination in conjunction with axon-glial contact also involved in the decrease in the Kv1.1 transcript?

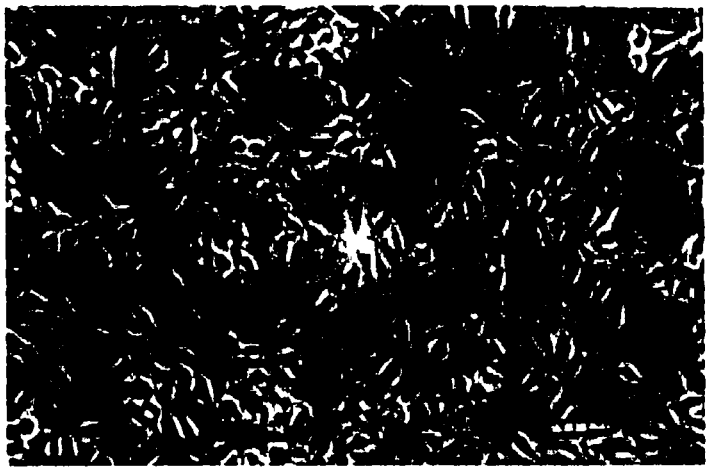
We have demonstrated in the previous chapter that in C6 Glioma, a cell line model system for an oligodendrocyte/astrocyte progenitor, that elevation of cAMP is strongly correlated with a decrease in expression of the delayed-rectifier K channel, Kv1.1. The results that we present in this chapter are consistent with work of others where it has been demonstrated that elevation of cAMP induces a morphological change and inhibits proliferation of C6 Glioma. Additionally, we have demonstrate that we can mimic the effects on morphology and proliferation with the K channel blocker 4AP. In contrast, while TEA blocks proliferation, there is a delay in the inhibition and there is no associated morphological change with this K channel blocker. We did not detect any inhibition of proliferation with the relatively specific Kv1.1 channel toxin, DTX-I. We are unable to determine from these data whether blocking Kv1.1 is sufficient for blocking proliferation due to the caveat that

we are not certain that the toxin remains intact long enough during the experiment to maximally block Kv1.1 channel activity. We tested the activity of the supernatants containing the DTX-I after 24 and 48 hours of incubation with the cells and determined that while the toxin in these culture supernatants was still active, it did not inhibit the $I_{K,D}$ to the same extent as fresh toxin. We postulate that it is possible that the toxin was partially degraded in culture and therefore there were enough 'active' Kv1.1 channels to maintain a control proliferation rate in the presence of DTX-I.

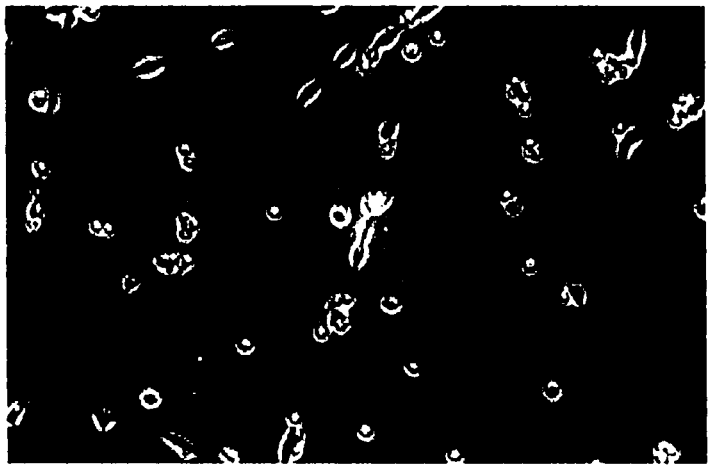
In conclusion, we present data that there is a strong correlation between the elevation of cAMP and down regulation of expression of Kv1.1 in C6 Glioma, and inhibition of proliferation. Furthermore, the results reported here show that elevation of cAMP and the K channel blocker 4AP induce morphological differentiation of C6 Glioma and inhibit proliferation. We speculate that tightly regulated expression of potassium channels may be a model for how cell differentiation is regulated in vivo. We speculate that expression of Kv1.1 is upregulated at stages that are permissive to proliferation and that under the proper cues, expression of Kv1.1 is down regulated prior to or coincident with differentiation.

Fig. 24. Potassium channel blockers inhibit proliferation and induce morphological changes in C6 glioma. These photomicrographs were taken on day 3 of treatments (same cultures used for proliferation assays Fig. 25). Iso/IBMX were used at 10μM/500μM, 4AP and TEA were each used at 5mM.

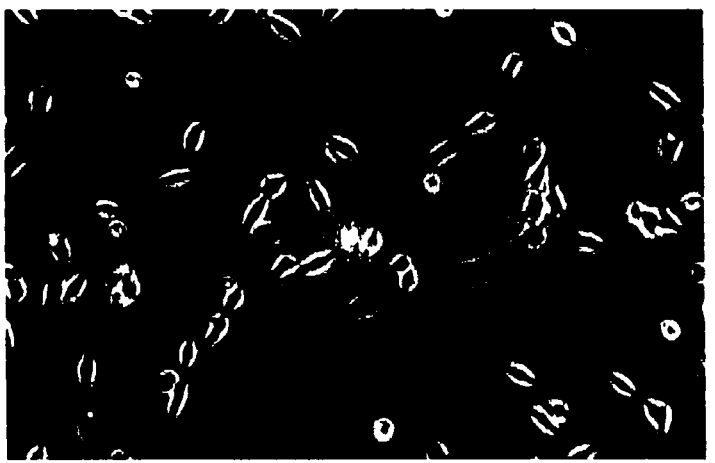
Control



Iso/IBMX



4AP



TEA

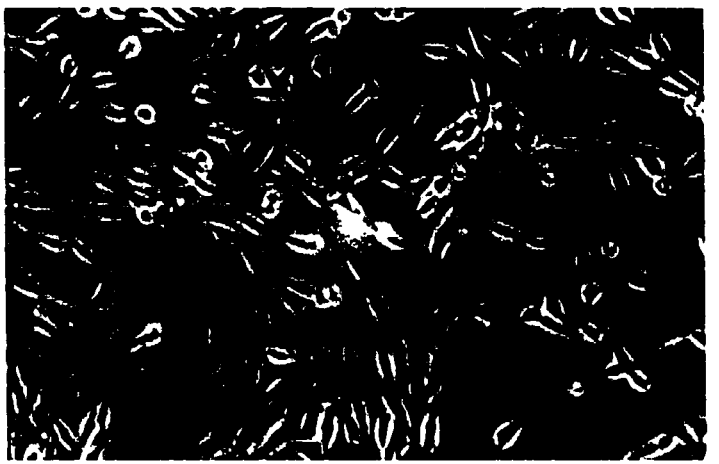


Fig. 25. Elevation of cAMP and potassium channel blockers inhibit proliferation of C6 glioma. **A.** Proliferation was assessed using cell counts. Three random fields were counted for each of three wells for each treatment. Error bars represent M.D. **B.** Proliferation was assessed by ^{3}H-Thymidine incorporation. The same plates used for **A.** were used for ^{3}H-Thymidine assays. All treatments were done in triplicate and error bars represent M.D. Cells were seeded at 1 x 10^{4} cells/well. 4AP and TEA were used at 5mM, Iso/IBMX were used at 10μM/500μM.

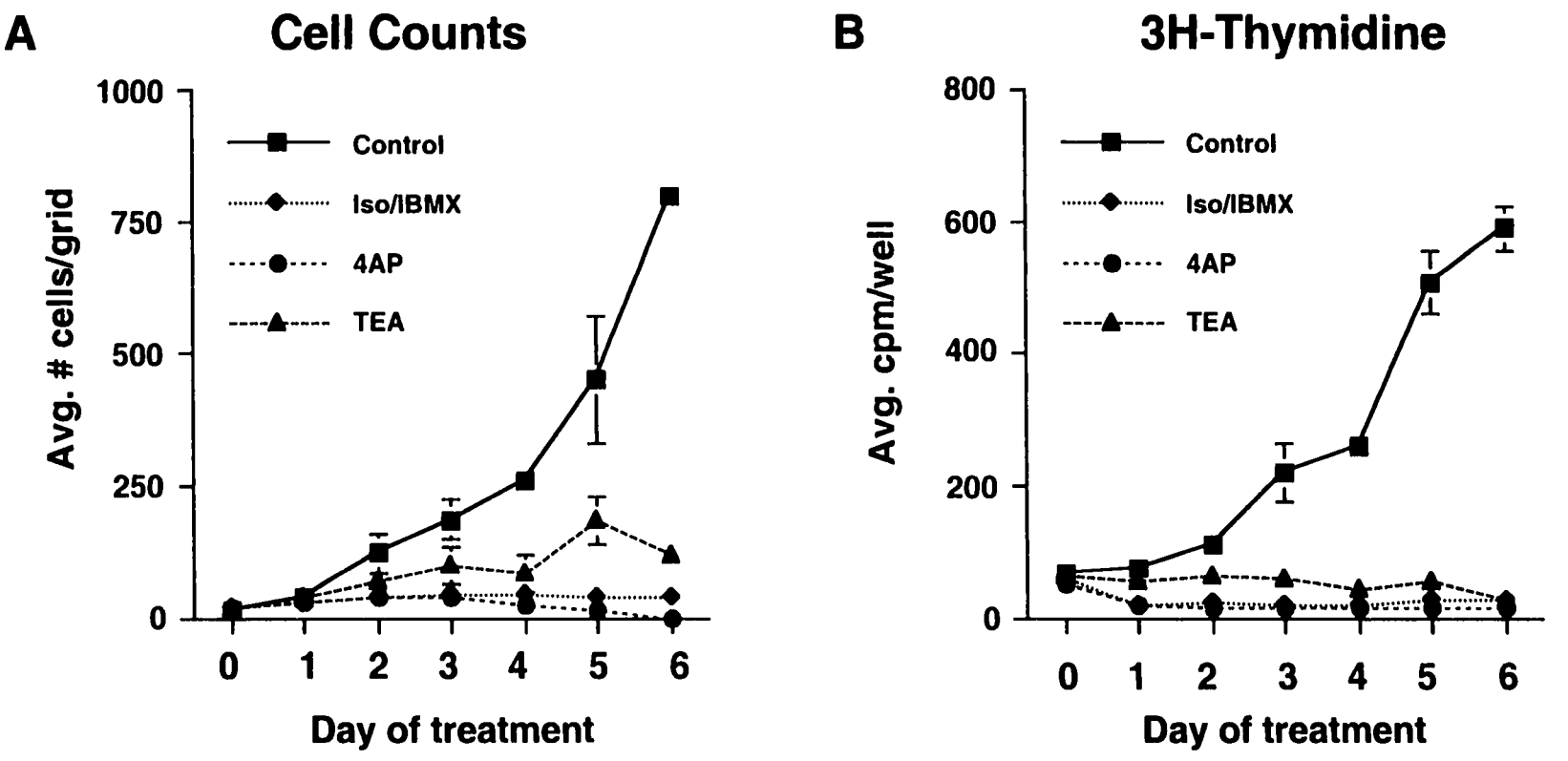
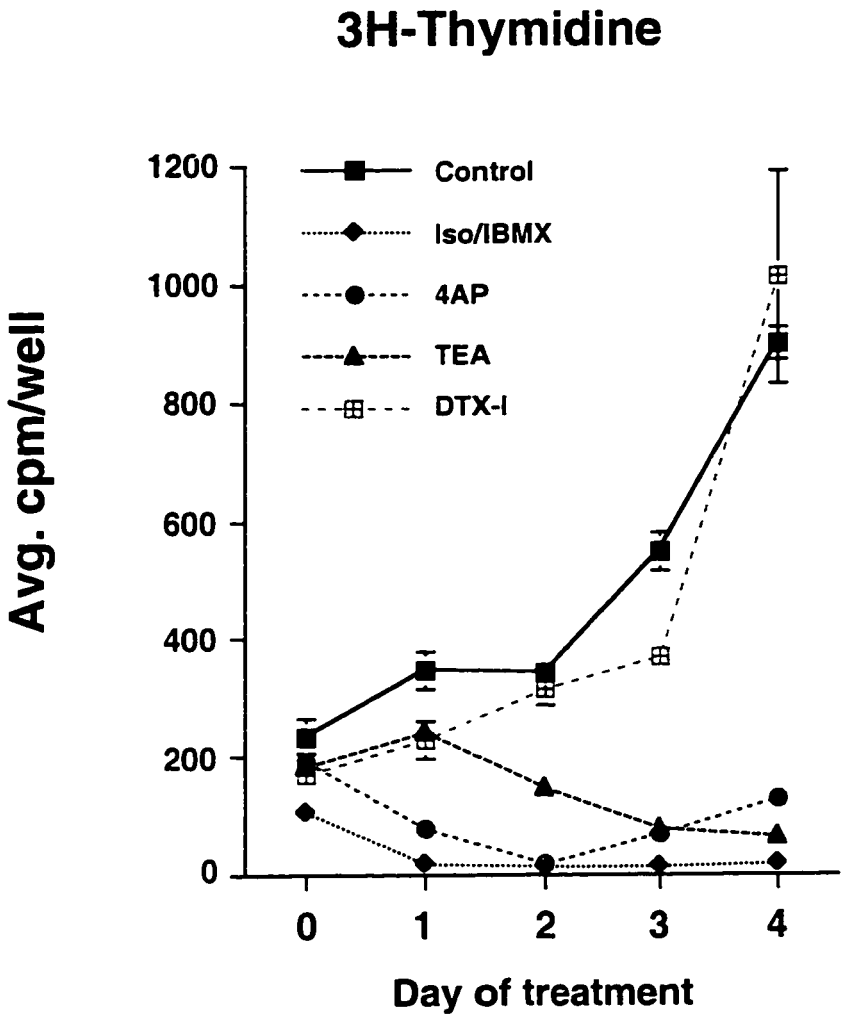

A
Cell Counts
Avg. # cells/grid
1000
750
500
250
0
Control
Iso/IBMX
4AP
TEA
0
1
2
3
4
5
6
Day of treatment
B
3H-Thymidine
Avg. cpm/well
800
600
400
200
0
Control
Iso/IBMX
4AP
TEA
0
1
2
3
4
5
6
Day of treatment

Fig. 26. Proliferation of C6 glioma inhibited by elevation of cAMP and the potassium channel blockers 4AP, TEA but not DTX-I. Proliferation was assessed by ^{3}H-Thymidine incorporation. All treatments done with n=4 and error bars represent M.D. Cells were seeded at 7.75×10^{4} cells/well. 4AP and TEA were each used at 5mM, Iso/IBMX were used at 10μM/500μM, DTX-I was used at 1μM.


3H-Thymidine
Avg. cpm/well
1200
1000
800
600
400
200
0
Control
Iso/IBMX
4AP
TEA
DTX-I
0
1
2
3
4
Day of treatment

SUMMARY

Potassium channels (K channels) have been identified in all cellular organisms - prokaryotes, plants, yeast and eukaryotes. K channels in mammalian organisms are important in maintaining cellular resting membrane potentials, in shaping action potentials, modulation of hormone or neurotransmitter release and regulation of neuronal firing patterns (Hille, 1992; Jan and Jan, 1997). For voltage gated K channels, the functional channel is composed of four alpha subunits and associated beta-subunits. The alpha subunits can associate as homotetramers or as heterotetramers - with the constraint that heterotetramers form only with members within a sub-family of alpha subunits. The electrophysiological and pharmacological parameters of the heterotetramers are unique and differ from those parameters of the homotetramers formed by the individual alpha subunits. In addition to the modulation of K channel function generated by heterotetramerization, function of K channels are regulated by other post-translational mechanisms including modification by sialic acid addition and phosphorylation. Although the voltage gated K channels are a large family with extensive overlap of expression patterns, perturbations of even one alpha subunit can have significant deleterious physiological effects. For example, point mutations in the open reading frame (ORF) of Kv1.1 which alter single

amino acid residues are responsible for the human disease episodic ataxia/myokymia syndrome. Furthermore, a mouse knock-out model in which Kv1.1 expression is ablated results in mice with severe epilepsy.

Since K channel function and the resulting effects on physiology can be significantly modulated by subunit composition, it is important to understand the mechanisms regulating expression of individual alpha subunits. The data presented here show that Kv1.1 transcription is consistent with TATAA-less, CCAAT-less, multiple transcriptional start sites. The adjacent proximal promoter region is sufficient to act as a basal promoter in luciferase expression constructs. The transcriptional start sites give rise to transcripts with a 5'UTR nearly 1kb in length and highly GC rich. Sequence comparison between mouse and human of the 5'UTR and proximal promoter region of Kv1.1 indicates a high degree of conservation (≥78%). Expression constructs with the 5'UTR of Kv1.1 fused with luciferase expression vector demonstrate that there is reduced expression of constructs with Kv1.1 5'UTR region. We postulate that the 5'UTR may confer translational regulation of expression of Kv1.1.

In addition to the primary sequence conservation, data is presented which demonstrates a correlation between elevation of cAMP and destabilization of Kv1.1 mRNA in the C6 glioma cell line. Subsequent to the destabilization in Kv1.1 mRNA there is a reduction in Kv1.1 protein

measured by Western blot analysis. There is a decrease in outward K current as measured by whole-cell patch clamp electrophysiology that parallels the decrease in Kv1.1 protein detected by Western blot analysis. The presence Kv1.1 in C6 glioma is thought to regulate the resting membrane potential (RMP). It is postulated that this mechanism of transcript destabilization may allow for a more rapid response in modulating Kv1.1 expression which in turn would alter K channel function thus modulating physiological responses to stimuli which signal through the cAMP second messenger cascade.

Finally, we present preliminary data which would suggest that in fact expression and/or function of Kv1.1 is important for proliferation of C6 glioma. We present data which demonstrates that the conditions which destabilize the Kv1.1 transcript and result in a reduction of Kv1.1 protein and decreases in outward K current also result in complete inhibition of proliferation of the C6 glioma. The general K channel blockers, 4AP and TEA can mimic these results suggesting that functional K currents are necessary for proliferation of C6 glioma. A correlation between outward K currents and proliferation has been described by Chiu and Wilson (1989) for Schwann cells. Hallows and Tempel (1998) have done developmental expression studies of Kv1.1 and describe expression in E14.5 embryos of Kv1.1 in radial glia, precursors of myelinating Schwann cells. In adult myelinated axons Kv1.1 is

found in the axon at juxtaparanodal regions of Nodes of Ranvier and not in Schwann cells (Mi et al., 1995; Wang et al. 1995). We postulate that Kv1.1 is expressed in proliferating Schwann cell precursors and downregulated with myelination - a process which can be initiated in vitro by elevation of cAMP. Studies are currently underway to determine if Kv1.1 knock-out mice are deficient in myelination.

In summary, this dissertation describes the 5′ structure of the Kv1.1 gene and mRNA; describes a correlation between elevation of cAMP and destabilization of Kv1.1 mRNA in C6 glioma with a corresponding reduction in Kv1.1 protein and reduction in outward K currents; and describes preliminary work to show that expression/function of Kv1.1 is important for proliferation of C6 glioma.

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IMAGE EVALUATION
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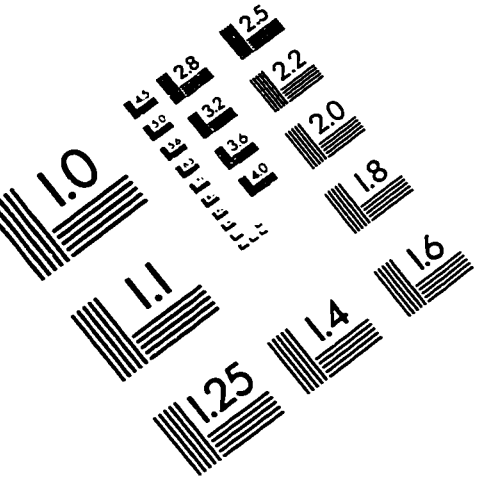

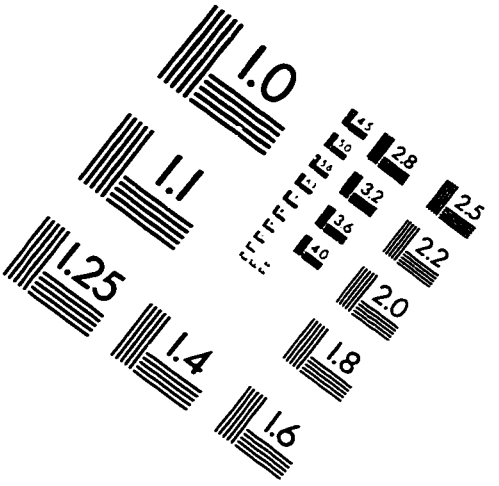

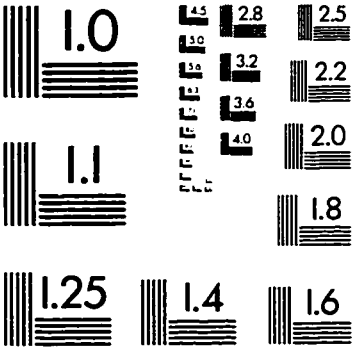

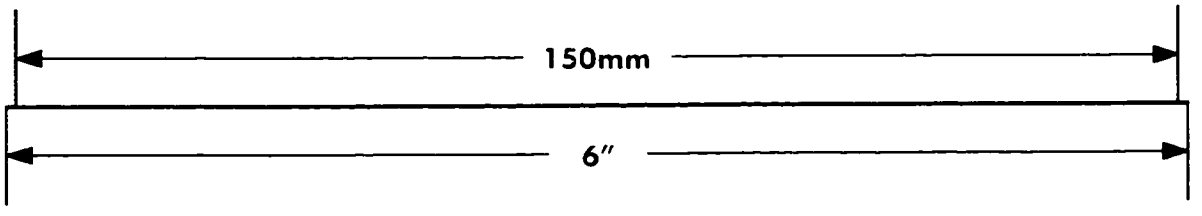

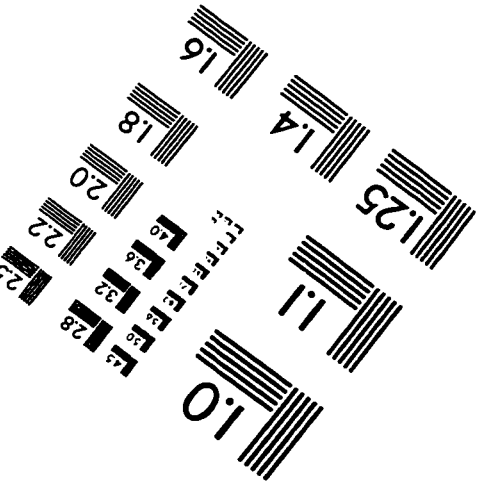

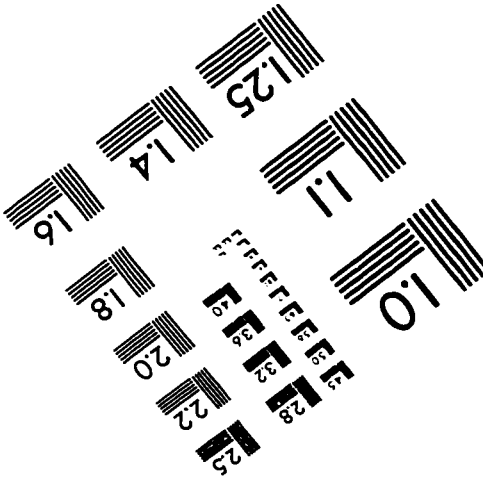

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