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Molecular Basis of Vascular Smooth Muscle Potassium Channels

By

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ABSTRACT

Vascular smooth muscle (VSM) cells wrap circularly around the endothelial cell monolayer that lines the blood vessel wall, functioning to control vascular tone, blood vessel diameter and blood pressure by changes in their contractility. K^+ channels, present within VSM cells, play a critical role in the control of contractility by regulating VSM membrane potential. Knowledge of the molecular basis of VSM K^+ channels provides important information for the development of therapeutic agents capable of modulating K^+ channel activity in the treatment of vascular diseases.

Specifically, this study provides evidence for the molecular basis of voltage-gated K^+ channels (Kv) and nucleoside diphosphate-sensitive K^+ channels (K^Dp) of VSM. The expression pattern of subunits known to form these two K^+ channel types was investigated in rabbit portal vein (RPV) VSM, utilizing reverse transcriptase-polymerase chain reaction to detect the expression of mRNA and subunit-specific antibodies to detect the presence of protein. In addition, the assembly pattern of subunit proteins underlying VSM Kv channels was probed by co-immunoprecipitation. Comparison of the biophysical and regulatory properties of recombinant channels, heterologously expressed in a mammalian cell line, to that of native currents provided functional evidence for the basis of VSM Kv and **KNJJP** channels.

Heteromultimeric Kv channel complexes composed of Kv1.2 and Kv1.5 subunits were concluded to underlie the slowly inactivating Kv current of RPV, based upon their co-immunoprecipitation and similar pharmacological and biophysical properties to the native current. A role of Kv0 subunits in forming VSM Kv channels was indicated by the co-immunoprecipitation of Kvpi.2 subunits with Kv1.5 subunits. Kv1.4-containing channel complexes were concluded to mediate the fast inactivating Kv current due to the biophysical similarity of Kv1.4 channels and the native current.

In addition, this study demonstrated the expression of mRNA encoding Kir6.1 and SUR2B subunits in RPV, subunits proposed to form the basis of VSM K-NDP channels. Furthermore, recombinant channels composed of these subunits demonstrated a similar inhibitory regulation by protein kinase C activation as native RPV **KNJ)P** channels, providing functional evidence that these subunits underlie VSM **KNDP** channels.

A C K N O W L E D G E M E N T S

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DEDICATION

I would like to dedicate this study to my wife Connie and to my three children Nicholas, Logan and Elle for their love and support.

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CHAPTER 1: INTRODUCTION

1.1. Blood pressure and vascular smooth muscle contractility

Human life is sustained by the flow of blood through blood vessels that serve to provide nutrients and remove waste products from all organs of the body. Control of blood flow to different areas of the body is, therefore, critical for regulating organ function. For example, increased pulmonary blood flow during physical activity is required in order to obtain sufficient oxygen for increased energy demands. Blood flow and pressure are a function of two primary factors: i) contractility of the heart, propelling blood through blood vessels, and ii) vascular resistance, determined by the overall vasoactive state of the body's blood vessels. Contractility of the heart and vascular resistance are both modulated by neuronal input, as well as by other locally released (e.g. endothelial factors) or circulating factors present within the blood. The influence of these factors facilitates a tight control of blood flow within specific regions of the body. Vasoconstriction of a blood vessel is a decrease in diameter that results in decreased blood flow, an increase in vascular resistance and increased blood pressure. Blood vessel vasodilation reduces vascular resistance in the opposite manner by increasing arterial diameter (Fig.l). In addition, resistance arteries exhibit myogenic reactivity, an intrinsic mechanism for the regulation of vascular resistance whereby modulation of arterial diameter is dependent on changes in local blood pressure. An increase in blood pressure causes myogenic vasoconstriction via the contraction of the V S M present in the arterial wall.

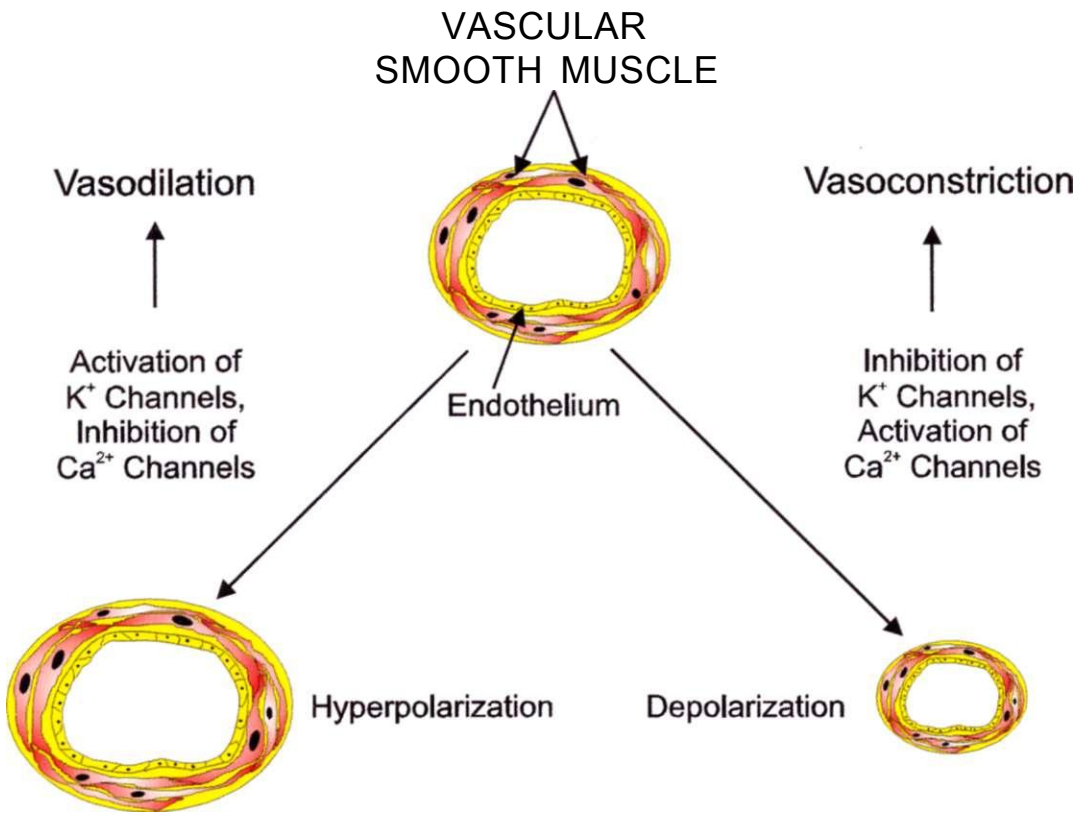


Figure 1: The role of membrane potential in the control of arterial tone and vascular resistance. The membrane potential of vascular smooth muscle is hyperpolarized by K^+ channel activity. This functions to limit Ca^{2+} influx through voltage-gated Ca^{2+} channels and favours vasodilation, whereas depolarization causes Ca^{2+} channel activation, Ca^{2+} influx and vasoconstriction, thereby decreasing arterial diameter.

The contractile state of V S M cells, which wrap circularly around the endothelial cell monolayer that lines the lumen of all veins and arteries (Fig. 1), is critical in dictating blood vessel diameter and, thereby, vascular resistance. V S M contractility is primarily regulated by changes in free cytoplasmic calcium concentration ([Ca^+]), whereby increases in cytoplasmic [Ca^+] cause contraction and vasoconstriction. ***Ca?***$^{+}$ causes contraction by binding to the protein calmodulin, which then activates myosin light chain kinase resulting in phosphorylation of myosin filaments, cross-bridge cycling and force development (Allen and Walsh, 1994) (Fig. 2). V S M relaxation and vasodilation is favoured by a decrease in cytoplasmic [Ca^+J and by the activity of myosin light chain phosphatase that dephosphorylates myosin, decreasing force.

1.2. Mechanisms regulating V S M cytoplasmic [Ca2+]

Increases in V S M cytoplasmic [Ca^+] occur via the release of ***Ca?***$^{+}$ from the sarcoplasmic reticulum (SR) and by the entry of Ca2+ into V S M cells from the extracellular space. [Ca^+J in the SR and the extracellular space is in the millimolar (mM) concentration range while cytoplasmic [Ca^+] ranges from 0.1-1 micromolar (uM), thereby providing a large chemical gradient for the movement of ***Ca?***$^{+}$ into the cytoplasm (Gollasch et al., 2000). C a^+ release from the SR occurs through ryanodine and inositol **1,** 4, **5-**trisphosphate **(IP3)** receptor channels embedded in the SR membrane that are activated by C a^+ and **IP3,** respectively. Cellular **IP3** is produced by the action of vasoactive agonists binding to sarcolemmal receptors, which lead to the activation of

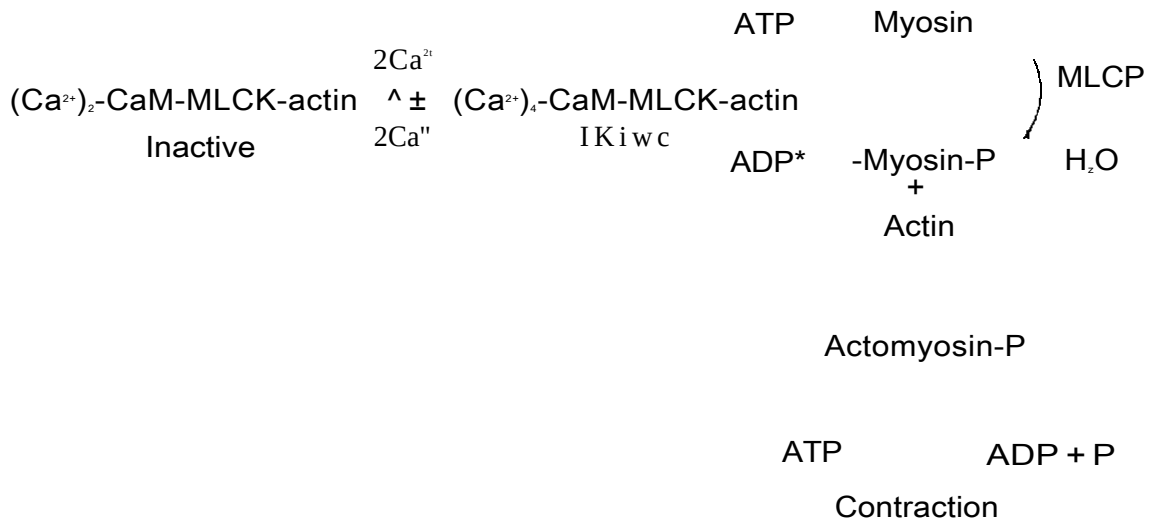


Figure 2: Control of smooth muscle contractility by Ca^{2+}. Increases in VSM cytoplasmic [Ca^{2+}] cause the activation of myosin light chain kinase (MLCK) by the increased binding of Ca^{2+} to calmodulin (CaM). MLCK phosphorylates myosin, activating cross-bridge cycling and contraction. Myosin light chain phosphatase (MLCP) dephosphorylates myosin and favours VSM relaxation.

phospholipase CP that hydrolyzes the membrane phospholipid phosphatidylinositiol 4, 5-bisphosphate (Lee and Severson, 1994). SR [Ca^+] is maintained by the action of SR pumps that function to move Ca^+ against its diffusion gradient from the cytoplasm into the SR at the expense of ATP hydrolysis (Marks, 1992).

Ca^+ entry into VSM cells from the extracellular space occurs in the most part through voltage-gated L-type Ca^+ channels that are activated by increased membrane potential (Em) depolarization with a threshold for activation of —30 mV (Nelson et al., 1990). The entry of Ca^4" through L-type channels results in an elevation of cytoplasmic [Ca^+J from -0.1 uM to concentrations as high as 1 uM. These increases in [Ca2+] result in activation of myosin light chain kinase and VSM contraction (Figs. 1 and 2). Em is a dynamic balance between inward (depolarizing) and outward (hyperpolarizing) currents across the VSM cell membrane, the sum of which results in a voltage difference between the extracellular and intracellular side of the membrane. Em is, therefore, critical to the control of L-type Ca^+ channel activity, VSM contractility and arterial diameter. L-type Ca^+ channels may also provide a source of 'trigger' ***Ca?***$^{+}$ for the activation of ryanodine receptors, releasing SR Ca^+ that augments the increase in cytoplasmic [Ca2+] and force of contraction (Gollasch et al., 2000).

Ca^+ entry from the extracellular space may also occur through non-selective cation channels that contribute to Em depolarization of VSM (Welsh et al., 2001). These channels have been implicated in the process of myogenic vasoconstriction based on their stretch-activated properties (Welsh et al, 2001; Wu and Davis, 2001). Non-selective

cation channels may provide the initial depolarization during increased luminal pressure, shifting V S M Em into the activation range for L-type ***Ca?***$^{+}$ channels, leading to C a ^ $^{+}$ entry and contraction.

1.3. Potassium channels in the control of Em and vascular tone.

It is well established that K^{+} channels carry the majority of V S M hyperpolarizing current and thus contribute to V S M relaxation by reducing L-type C a ^ $^{+}$ channel activity and the entry of contractile C a ^ $^{+}$ (Fig. 1) (Nelson and Quayle, 1995). This view is based on a large body of evidence utilizing pharmacological agents that manipulate K^{+} channel activity while measuring the force and diameter of V S M strips and pressurized intact arteries, respectively. Agonists leading to the activation or inhibition of K^{+} channels were found to result in arterial vasodilation and vasoconstriction, respectively. K^{+} channel openers, which selectively activate ATP-sensitive K^{+} channels, elicit relaxation and a number of selective K^{+} channel blockers have demonstrated contractile properties (Nelson and Quayle, 1995; Quayle et al., 1997).

1.4. Identification of vascular smooth muscle K^{+} channels.

Four different K^{+} conductances have been identified as modulators of V S M Em and contractility. These include: i) voltage-gated (Kv), ii) large conductance calcium-activated (BKca), iii) inward rectifier (Kir) and iv) ATP-sensitive (K^\jp) channels (Nelson and Quayle, 1995). These four V S M K^{+} conductances have unique biophysical

and pharmacological properties that are consistent with the divergent properties observed from the cloning and heterologous expression of K^+ channel genes from smooth muscle (Cole and Clement-Chomienne, 2000), as well as from other excitable cell types, such as cardiac myocytes and neurons (Seino, 1999; Nerbonne, 2000). The molecular basis of K^+ channels in VSM, and other excitable cell types, has been of great interest in an attempt to find targets for the control of different disease states, such as diabetes, hypertension, heart failure and stroke. The cloning of K^+ channel subunits from VSM, combined with their heterologous expression, enables a comparison of the biophysical properties of currents produced by cloned channel subunits to native VSM currents. This approach has provided functional evidence for the molecular components that underlie native VSM channels, yet differences in cloned channel properties are observed dependent on the expression system utilized, making comparative studies of biophysical properties difficult to interpret (Uebele et al, 1996; Petersen and Nerbonne, 1999). More substantial lines of evidence are required in order to determine the molecular basis of native VSM K^+ channels. These include: i) Evidence for the expression of channel **proteins** in the VSM of interest utilizing specific antibodies for channel subunits. ii) Similar pharmacology of cloned channels to that of native channels, iii) Suppression of native channels by genetic knock-out, or anti-sense oligonucleotides against specific channel subunit mRNAs. iv) Viral gene transfer resulting in the expression of dominant-negative channel proteins that result in loss of native channel function, v) Immunoprecipitation of channels with subunit-specific antibodies to enable determination of the subunit composition of native channel complexes, vi) Demonstration that cloned

channels in heterologous expression systems exhibit regulation by vasoactive agonists similar to that documented for native V S M channels.

1.4.1. Voltage-gated K^+ channels

1.4.1.1. Voltage-gated K^+ channels in the control of vascular tone.

Voltage-gated K^+ channels (Kv) contribute to the maintenance of V S M Em and vascular tone, as demonstrated through the use of the selective Kv channel blocker 4-aminopyridine (4-AP). 4-AP has demonstrated an ability to depolarize V S M Em and constrict intact pressurized arteries (Leblanc et al., 1994; Knot and Nelson, 1995). Moreover, V S M Kv currents recorded from isolated vascular myocytes (Beech and Bolton, 1989a, 1989b; Volk et al, 1991; Robertson and Nelson, 1994; Nelson and Quayle, 1995; Aiello et al, 1995, 1996, 1998; Clement-Chomienne et al, 1996, 1999; Remillard and Leblanc, 1996), and from heterologous expression systems transfected with Kv channels expressed in V S M (Po et al, 1993; Clement-Chomienne et al., 1999; Kerr et al, 2001), demonstrate activation at voltages consistent with the Em reported for V S M cells of pressurized intact arteries, i.e. -40 to -55 mV (Knot and Nelson, 1995; Nelson and Quayle, 1995). V S M 4-AP-sensitive Kv channels have also demonstrated regulation of channel activity by vasoactive agonists coupling to protein kinase A (PKA) and protein kinase C (PKC) (Aiello et al., 1995, 1996, 1998; Clement-Chomienne et al., 1996; Shimoda et al., 1998). Furthermore, 4-AP-sensitive Kv channels have been implicated in hypoxic pulmonary vasoconstriction (reviewed by Kozlowski, 1995 and Yuan et al., 1995; Archer et al., 1998; Hulme et al., 1999; Platoshyn et al, 2001). The constriction of pulmonary arteries in response to hypoxia results in a diversion of blood

to better ventilated areas of the lung. This phenomenon is unique to the pulmonary bed, as other systemic vascular beds dilate, or display no change in arterial diameter, in response to hypoxia (reviewed Kozlowski, 1995). These differences in response to hypoxia may result from differences in the regulation and/or expression of specific Kv channel subunits in different vascular beds. Pulmonary Kv currents have demonstrated inhibition by acute hypoxia (Archer et al, 1996) and decreased channel density is observed following chronic hypoxia, conditions also exhibiting a coincident increase in basal cytoplasmic [Ca^+J (Platoshyn et al., 2001). A decrease in pulmonary myocyte Kv current has also been implicated in patients with primary pulmonary hypertension (Yuan et al., 1998a, 1998b). These data demonstrate a fundamental role for Kv channels in regulating vascular tone and blood pressure.

1.4.1.2. Vascular smooth muscle Kv current properties and 4-AP sensitivity.

V S M Kv currents have been recorded from myocytes isolated from a number of different vessels including portal vein, cerebral, coronary, mesenteric, pulmonary and renal arteries (Beech and Bolton, 1989a, 1989b; Volk et al., 1991; Gelband and Hume, 1992; Smirnov and Aaronson, 1992, 1996; Robertson and Nelson, 1994; Leblanc et al., 1994; Aiello et al., 1995; Archer et al, 1996; Remillard and Leblanc, 1996; Clement-Chomienne et al., 1999; Lu et al., 2001). Two components of V S M Kv currents have been described: i) a slowly activating and inactivating delayed rectifier current (Kdr) that displays little or no inactivation during 250 ms step depolarizations and, ii) a fast activating and inactivating transient outward current (Kto) that activates and inactivates within 10-50 ms. Kdr currents have been observed in all vascular myocytes studied to

date, whereas Kto currents have only been observed in myocytes isolated from a small number of vessels, such as portal vein, aorta and pulmonary artery (Beech and Bolton, 1989a; Halliday et al., 1995; Shimoda et al., 1998). A predominance of Kto current has been documented in cultured pulmonary artery myocytes (Yuan, 1995; Yuan et al., 1998a), perhaps due to a change in Kv channel expression under these conditions (Tang and Wang, 2001).

Considerable diversity in V S M Kv current density, pharmacology and biophysical properties has been reported. This diversity may perhaps be attributed to vessel-specific differences in the expression and assembly pattern of Kv channel subunits. On the other hand, the low density of whole-cell V S M Kv current reported in many studies is consistent with damaged or contracted myocytes resulting from poor isolation techniques. Therefore, differences in reported channel properties in myocytes isolated from different vessels may be a function of the efficiency of cell isolation, resulting in a variable loss of different channel populations that contribute to net whole-cell Kv current. The steady-state activation and inactivation properties, and the reported **IC50** values for 4-AP inhibition of Kdr recorded from different vascular myocytes are summarized in Table 1. Reported **IC50** values for 4-AP block of V S M Kdr are generally 1 m M or less. It is difficult to make a comparison of the values in Table 1 due to differences in cell isolation and/or recording conditions utilized for these studies. However, a comparison of Kdr currents can be made for rat cerebral artery (Iftinca and Cole, unpublished data), rat mesenteric artery (Plane and Cole, 2000), rabbit coronary artery (Sontag and Cole, unpublished data) and rabbit portal vein (RPV) (Clement-Chomienne et al., 1999), as

Table 1: VSM whole-cell Kv current 4-aminopyridine (4-AP) sensitivity, and steady-state activation and inactivation properties.

Vessel/Species	**Activation** $V_{0.5}$(mV),k (mV)	**Inactivation** $V_{0.5}$ (mV), k (mV)	**4-AP IC_{50}** (mM)	**Reference**
cerebral/rabbit	-10, 11	-44, 6.6	<1	Robertson and Nelson, 1994
cerebral/rat	-20, ND	-17.8, ND	ND	Iftinca and Cole, unpublished data
coronary/rabbit	-16, 9.4	-37, 8.0	1.37	Remillard and Leblanc, 1996
coronary/rabbit	-5.6, 8.7	-24.2, 5.2	ND	Volk et al, 1991
coronary/rabbit	-14.9, 6.1	-33.3, 8.2	0.31	Sontag and Cole, unpublished data
mesenteric/rat	-10.8, ND	-31.0, ND	ND	Plane and Cole, 2000
mesenteric/rat	-11, 15	-40, 14	5.1	Luetal., 2001
mesenteric/ human	ND	ND	1.16	Smirnov and Aaronson, 1992
portal vein/ rabbit	-16.1, 8	-36.4, 7	0.356	Clement-Chomienne et al., 1999
pulmonary/ rabbit	ND	ND	0.3	Okabeetal., 1987
pulmonary/ canine	-23.0, 6.3	ND	ND	Ann and Hume, 1997
pulmonary/rat	ND	-13.9, 9.1	ND	Smirnov and Aaronson, 1996
renal/rabbit	-14.9, 6.5	ND	ND	Prior etal, 1998
renal/canine	ND	ND	0.723	Gelband and Hume, 1992

ND = not determined

identical recording conditions were utilized for all four of these studies, facilitating a vessel-to-vessel comparison of VSM Kdr properties. The biophysical properties and 4-AP sensitivities of rabbit coronary artery and RPV Kdr are similar. On the other hand, cerebral artery Kdr is the most different of these four Kdr currents, demonstrating activation at more negative voltages and inactivation at substantially more positive voltages than RPV or coronary artery Kdr, suggesting a larger range of steady-state Kdr window current in cerebral than in RPV, coronary and mesenteric artery myocytes. Rat mesenteric artery Kdr activates more positively than the Kdr of all other vessels, with inactivation properties close to those of RPV and coronary artery. This indicates that mesenteric Kdr displays the smallest range of steady-state Kdr window current of all four of the VSM Kdr currents.

Several studies indicate that the Kdr current of coronary, mesenteric and RPV vascular myocytes is composed of at least two components, a 4-AP-sensitive Kdr current and a 4-AP-insensitive Kdr current (Beech and Bolton, 1989b; Remillard and Leblanc, 1996; Clement-Chomienne et al, 1999; Kerr et al, 2001; Lu et al., 2001). The 4-AP-sensitive Kdr current is the major determinant (-70%) of Kdr current in these vascular myocytes, which demonstrates a shift in steady-state activation coincident with 4-AP block (Remillard and Leblanc, 1996; Plane and Cole, 2000; Kerr et al, 2001).

1.4.1.3. The Kv channel superfamily

The Kv channel superfamily is composed of six-transmembrane domain pore-forming alpha (a) subunit families Kv1-Kv4 (Fig. 3A), in which members from the same family co-assemble in a homo- or hetero-tetrameric manner to form channel complexes

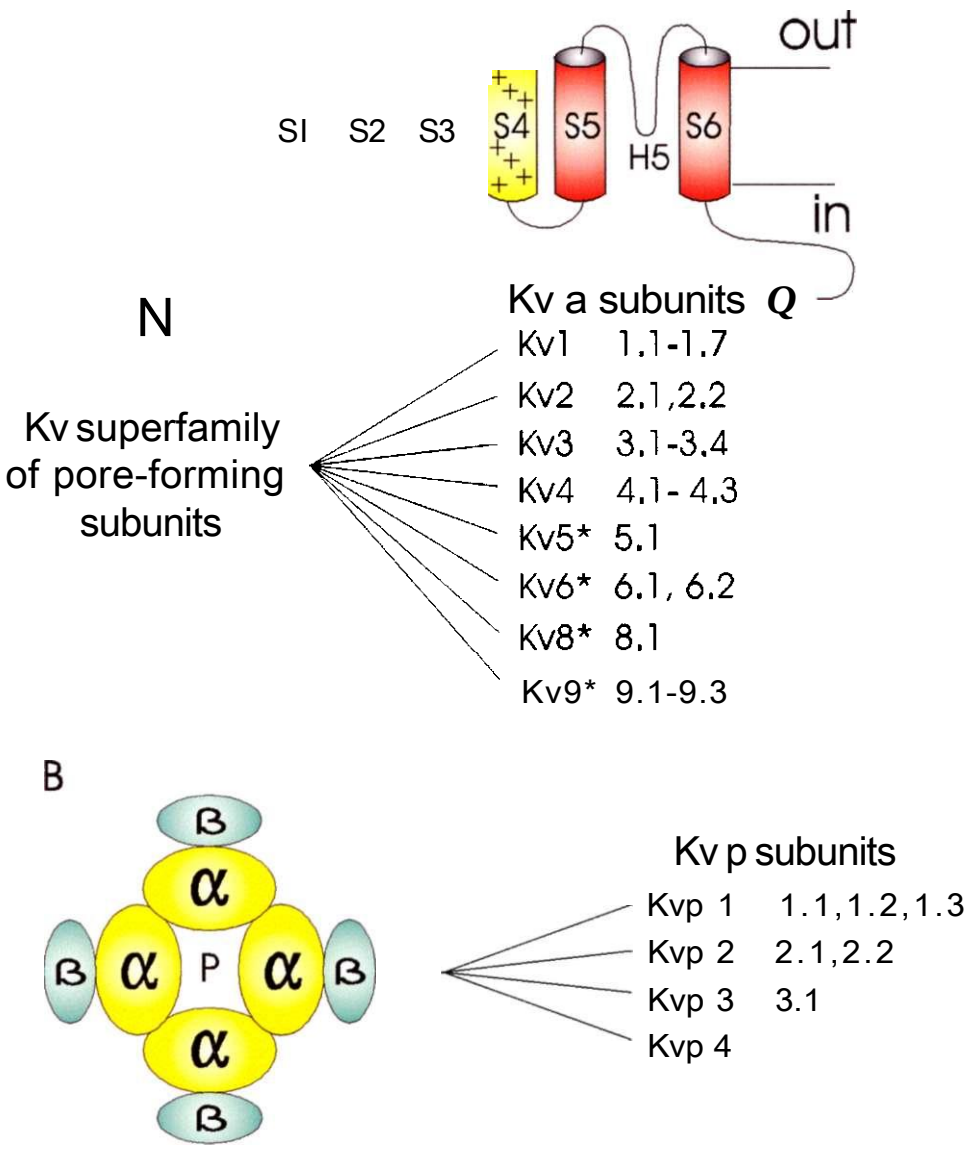


Figure 3: Proposed topology and assembly of Kv channels. A: Topology of pore-forming Kva subunit super family comprised of Kvl-Kv9 families. * denotes subunits forming electrically silent homomultimeric channels that function to modulate Kv2 and Kv3 channels by heteromultimeric assembly. **B:** Four Kv a subunits within the same family co-assemble with four Kvp subunits in a functional Kv channel complex. Kvp subunit family is comprised of Kvpi-4 with splice variants of Kvp 1 and Kvp2.

with differing properties (Robertson, 1997; Nerbonne, 2000). Kv5, Kv6, Kv8 and Kv9 a subunit families do not form functional channels when expressed alone, but co-assemble with a subunits of Kv2 or Kv3 to produce channel complexes with distinct properties from those of Kv2 and Kv3 homomultimers (Salinas et al., 1997; Kramer et al., 1998). The large diversity of Kv channels is further increased by modulatory Kvbeta (Kvf3) subunits that function to co-assemble with the pore-forming Kvoc subunits, modulating biophysical properties and channel trafficking to the membrane (reviewed by Xu and Li, 1998; Trimmer 1998; Pongs et al., 1999). The Kvf3 subunit family includes Kvpi-4, with splice variants of the Kvpi and Kvp2 genes (Xu and Li, 1998; Pongs et al., 1999) (Fig. 3B). The precise assembly pattern of Kva and Kvp subunits into functional channel complexes can, therefore, provide unique properties catered to tissue-specific requirements for cellular function.

1.4.1.4. Kv channel crystal structure.

Recently, the three-dimensional crystal structure of the bacterial K^+ channel KcsA was determined (Doyle et al., 1998). Each subunit of the KcsA channel contains two transmembrane domains separated by a H5 domain or pore loop, analogous to the proposed topology of pore-forming inward rectifier K^+ channel subunits (Fig. 4; see below), and that of the S5 through S6 domain of Kv channels (Fig. 3). The KcsA crystal structure demonstrated a tetrameric assembly pattern of channel subunits, with each of the four subunits contributing a H5 domain to form the K^+ selectivity filter within the pore of the channel. The intracellular ends of the transmembrane domains were found to

be positioned angling toward the center of the channel, forming the bottom of an inverted 'tee-pee'-like structure inserted into the membrane.

The KvP subunit structure, along with the structure of the a:P assembly domain, has also been determined by crystallography (Gulbis et al., 1999, 2000). KvP subunits form a tetrameric structure located at the cytoplasmic side of the membrane and bound to the Tl domain of the Kva subunit, which is located within the cytoplasmic amino terminus (Gulbis et al., 2000). Interestingly, the Kvp subunit crystal structure and amino acid sequence indicate that these proteins are members of the NADH-dependent oxido-reductase family of enzymes (McCormack and McCormack, 1994; Gulbis et al, 1999). This suggests that KvP subunits may play a role in linking the redox status of the cell to Kv channel function.

1.4.1.5. Molecular identity of VSM Kv channels.

The molecular identity and assembly pattern of Kv subunits into functional channel complexes in smooth muscle remain to be established, unlike neuronal Kvl channel complexes that have been well defined in a number of species (Rhodes et al., 1997; Shamotienko et al., 1997; Coleman et al, 1999). Previous studies suggest a complex expression pattern of Kv subunit genes in smooth muscle, and the possibility of differential expression of Kv subunit genes in different vascular beds. In VSM of portal vein, carotid, mesenteric, pulmonary and tail arteries numerous mRNA transcripts for different Kv channel subunits have been detected (Yuan et al., 1998c; Clement-Chomienne et al., 1999; Hulme et al., 1999: Xu et al., 1999, 2000; Osipenko et al., 2000; Ohya et al., 2000; Cox et al., 2001; Lu et al., 2001). Similarly, airway and gastrointestinal

smooth muscles have demonstrated the expression of numerous Kv subunit mRNA transcripts (Adda et al., 1996; Wade et al., 1999; Epperson et al., 1999). Significantly, a number of Kv subunits of the gastrointestinal tract demonstrate differences in expression dependent on the region of smooth muscle analyzed (Epperson et al, 1999); these differences may contribute to the varied electrical activity observed in different areas of this tissue.

However, the studies on VSM Kv subunit expression have been difficult to interpret due to: i) The use of cultured smooth muscle cells for mRNA detection by RT-PCR and immunocytochemical detection of Kv subunits (Yuan et al, 1998; Xu et al., 1999, 2000), conditions which are known to alter not only the phenotype of VSM cells but K^+ channel expression as well (Tang and Wang, 2001), ii) The use of whole tissues for mRNA detection without confirmation of protein expression within isolated vascular myocytes (Ohya et al, 2000; Cox et al., 2001). This leaves the possibility of detection of Kv subunits from non-smooth muscle cell types such as neurons and lymphocytes, iii) A lack of control experiments to demonstrate the specificity of antibody immunoreactivity observed in protein detection experiments (Archer et al., 1998; Yuan et al., 1998). An approach utilizing not only RT-PCR for mRNA detection of specific subunits, but the detection of expressed proteins within vascular myocytes utilizing antibodies with demonstrated specificity for individual subunits is lacking. In addition, the assembly pattern of Kva and Kvf3 subunits into VSM Kv channel complexes has not yet been addressed. This is significant, as the majority of neuronal Kv channels display heteromultimeric assembly (Rhodes et al., 1997; Shamotienko et al, 1997; Coleman et

al, 1999), which provides for unique functional properties not displayed by the homomultimeric assembly of the same channel subunits (Po et al., 1993; Kerr et al., 2001).

1,4.1.6. Molecular identity of 4-AP-sensitive Kv channels of RPV

The 4-AP-sensitive Kdr of RPV vascular myocytes exhibits an IC_{50} value for 4-AP of ~ 200 uM (Clement-Chomienne et al., 1999; Kerr et al, 2001). The 4-AP sensitivity and biophysical properties of this current are similar to those of Kdr recorded from other vascular myocytes (Table 1), and are consistent with the properties of Kvl members of the Kv channel superfamily (Grissmer et al, 1994; Lee et al, 1996; Uebele et al, 1996; Clement-Chomienne et al, 1999; Judge et al, 1999), and not with those of Kv2-Kv4 family members (Kirsch and Drewe, 1993; Critz et al, 1993; Grissmer et al., 1994; Kanemasa et al, 1995; Fiset et al., 1997; Schmalz et al., 1998; Franqueza et al., 1999). Reported IC_{50} values for 4-AP block, or concentrations of 4-AP demonstrating 50% inhibition, of members of the Kv channel superfamily have been in the following ranges: Kvl ~ 100-800 uM, Kv2 ~ 1-3 mM, Kv3 ~ 10-40 pM, and Kv4 ~ 1-4 mM (Kirsch and Drewe, 1993; Grissmer et al., 1994; Kanemasa et al, 1995; Fiset et al, 1997; Schmalz et al., 1998; Clement-Chomienne et al, 1999; Kerr et al, 2001). Heterologous expression of Kv2 family members demonstrates whole-cell currents with Kdr properties. Kv2 currents are slower to activate upon step depolarizations and are activated at a slightly more positive threshold than RPV Kdr current (Kirsch and Drewe, 1993; Schmalz et al., 1998). Members of the Kv3 family demonstrate whole-cell currents with a significantly more positive activation threshold (~ -10 mV) than RPV Kdr current. Some

members of the Kv3 family demonstrate fast and others slow inactivation kinetics (Critz et al., 1993; Kanemasa et al., 1995; Osipenko et al., 2000). Kv4 family members have fast inactivating properties with a threshold for activation of ~ -40 mV (Fiset et al., 1997; Franqueza et al., 1999). Therefore, Kv3 and Kv4 fast inactivating subunits are inconsistent with the biophysical properties of the Kto current that demonstrates a threshold for activation of —65 mV (Beech and Bolton, 1989a). Therefore, it can be concluded that Kvl channels are the dominant Kv channels responsible for RPV Kv current, with at most a minimal contribution from Kv2-Kv4 family members. A role of Kv2-Kv4 family members in V S M Kv current of other vessels is indicated by evidence supporting a role of Kv2 and Kv3 channels as mediators of hypoxic vasconstriction of pulmonary arteries (Patel et al., 1997; Osipenko et al, 2000).

It was demonstrated previously that Kvl.5 cloned from RPV and expressed in a mouse fibroblast cell line (L cells) produced a Kv current with properties similar to those of the Kdr current of RPV myocytes (Clement-Chomienne et al., 1999). Yet, properties of 4-AP-sensitive Kv current of RPV are inconsistent with Kvl.5 as the sole subunit underlying the current. These include: i) distinct rates and voltage dependence of inactivation (Clement-Chomienne et al, 1999), ii) lack of regulation of Kvl.5 channels by PKA (Thorneloe et al., 2000), and iii) differing effects on voltage dependence of activation produced by 4-AP block (Kerr et al, 2001). Therefore, it is reasonable to propose that other Kvla and/or KvP channel subunits may be part of the 4-AP-sensitive Kv current in RPV, consistent with the expression of multiple Kvl channel subunits in

other vascular beds (Archer et al, 1998; Yuan et al, 1998c; Hulme et al, 1999: Xu et al., 1999, 2000; Ohya et al., 2000; Cox et al, 2001).

1.4.2. Calcium-activated, large-conductance K^+ channels (BK_{Ca})

1.4.2.1. BK_{Ca} channels in the control of vascular tone.

BK_{Ca} channel activity has been recorded from all types of smooth muscle myocytes, as well as from a variety of other cell types, such as neurons (Nelson and Quayle, 1995). BK_{Ca} channels are voltage-gated channels of ~ 200 pS in conductance that are activated by increases in local [Ca^+j at the cytoplasmic face of the channel. Ca^{2+} activates BK_{Ca} channels in a concentration-dependent manner, by shifting the steady-state activation curve to more negative membrane potentials that fall within the physiological range of VSM Em during a sufficient Ca^{2+} transient (Cox and Aldrich, 2000). The importance of BK_{Ca} channels in controlling smooth muscle myogenic tone and contractility has been established through the use of selective toxins, such as charybdotoxin and iberiotoxin, that have demonstrated constriction of intact pressurized arteries (Brayden and Nelson, 1992; Nelson et al., 1995). As described above, resistance arteries develop myogenic tone in response to increased intra-luminal pressure, resulting from membrane depolarization, L-type Ca2+ channel activation and increased cytoplasmic [Ca^+J. These are conditions that also favour BK_{Ca} channel activation. Therefore, BK_{Ca} channels function in a negative feeback mechanism on VSM membrane depolarization and Ca^{2+} entry by hyperpolarizing Em. Consistent with this theory, the vasoconstrictor effects of iberiotoxin and charybdotoxin are decreased at lower intra-

luminal pressures and during block of L-type Ca^+ channels to limit the entry of extracellular Ca^+ (Brayden and Nelson, 1992). More recently, it has been documented that BK£ₐ channels are activated by Ca^+ sparks, which are localized increases in cytoplasmic [Ca^+J due to SR Ca^+ release through ryanodine receptor channels (Jaggar et al, 2000). ***Ca?***⁺ sparks cause a coincident and transient increase in BK^ₐ channel activity, first described as spontaneous transient outward currents (STOCs) (Benham and Bolton, 1986). A coupling of Ca^+ sparks and BK^ₐ channels enables the delivery of Ca^+ to the channels at a sufficient localized concentration (~ 1 uM) to shift the steady-state activation of BK^ₐ channels into the range of VSM Em, resulting in channel activation.

1.4.2.2. Molecular basis of VSM BK^ₐ channels

Structurally, the BK^ₐ channel pore-forming subunit is similar to Kv channel a subunits with a pore-forming H5 domain and a voltage sensor located in the fourth transmembrane domain (Fig. 3). However, BK^ₐ channels have also been shown to contain an additional transmembrane domain, SO, positioning the N-terminus of the channel at the extracellular side of the plasma membrane (Wallner et al., 1996). Ca^+ modulates BK£ₐ channel function via Ca2⁺-binding domains that are present in the long cytoplasmic carrx)xy-terminus of the channel (Braun and Sy, 2001). Only one pore-forming subunit gene has been discovered to date, producing a number of splice variants that may partially explain BK£ₐ channel diversity (Vogalis et al., 1996). Diversity is also

provided by at least 3 different BKc$_a$ (3 subunit families that co-assemble with the pore-forming subunit via an interaction with its SO domain (Meera et al., 1997). As described for Kvp subunits, BK^$_a$ p subunits function to modulate the biophysical properties of channel complexes. For example, differences in ***Ca***^$^+$ sensitivity and inactivation kinetics are observed, depending on the channel subunit composition (Uebele et al., 2000). Specifically, the BKc$_a$ pi subunit is highly enriched in smooth muscle tissues (Jiang et al., 1999) and functions to increase the Ca^$^+$ sensitivity of the channels in this tissue (Brenner et al., 2000; Cox and Aldrich, 2000). The dominant role of the BKc$_a$ pi subunit in coupling ***Ca***^$^+$ sparks to the BK^$_a$ pore-forming subunit, and in controlling VSM contractility, was recently shown by two independent groups via the use of BKca PI subunit knock-out (KO) mice (Brenner et al., 2000; Pluger et al., 2000). Lower levels of STOC activity were observed in KO mice and the contribution of BK£$_a$ channels to the control of VSM tone was also reduced, consistent with a decreased Ca^+ sensitivity of the BKc$_a$ channel due to the lack of the BKc$_a$ pi subunit.

1.4.3. Inward Rectifier K$^+$ Channels (Kir)

1.4.3.1. VSM Kir channels and control of vascular tone.

Of the four major VSM K$^+$ conductances identified, Kir channels have been the least studied. Inward rectifier currents have been described in myocytes isolated from small arteries and in short segments of intact arterioles (Edwards et al., 1988; Quayle et al., 1993, 1996). In general, Kir currents have demonstrated decreased density with increasing vessel size (Quayle et al., 1996) and an absence from large conduit arteries

(Cole and Clement-Chomienne, 2000; Nelson and Quayle, 1995). VSM Kir currents demonstrate strong inward rectification, with only a small outward current recorded just positive to the equilibrium potential for K^+ (Ek ~ -85 mV, under physiological conditions), and a large amount of inward current negative to Ek (Quayle et al, 1997; Bradley et al, 1999). The property of inward rectification has been shown to be a combination of intrinsic channel properties combined with internal block by $Mg2^+$ and polyamines (Ruppersburg, 2000). Therefore, current carried by VSM Kir channels under physiological K^+ gradients in the range of VSM Em (-60 to -30 mV) is minimal. A potential physiological importance of VSM Kir channels has been demonstrated with the use of small increases in external potassium, up to 15 mM, conditions that occur physiologically during hypoxia, ischemia or hypoglycemia (Knot et al, 1996; reviewed by Quayle et al., 1997). The increase in external potassium causes a positive shift in Ek, resulting in an increase in outward current occurring in the range of VSM Em. This effect has been documented by the effect of changes in external $[K^+]$ on VSM Kir currents from isolated cerebral artery myocytes (Quayle et al., 1993, 1996). This small amount of outward current, due to increased external $[K^+]$, is sufficient to cause a significant hyperpolarization of the VSM Em, due to the high input resistance characteristic of VSM cells (Nelson and Quayle, 1995; Cole and Clement-Chomienne, 2000). This, therefore, results in a K^+-induced vasodilation.

1.4.3.2. Molecular basis of VSM Kir channels

Cloning experiments of Kir channels from various tissues have identified the presence of 6 Kir families (Kir1-6). Expression of these 6 families in heterologous

expression systems has demonstrated members of the KM and Kir3-6 families to have weak inward rectification properties (Quayle et al., 1997; reviewed by Cole and Clement-Chomienne, 2000). In contrast, channels due to the Kir2 family have demonstrated strong inward rectification that is consistent with native VSM Kir currents recorded from myocytes of small arteries and segments of arterioles (Edwards et al, 1988; Quayle et al, 1993, 1996; Nelson and Quayle, 1995). The proposed topology of Kir subunits suggests two transmembrane domains (Ml, M2), with the amino and carboxy termini located on the cytoplasmic side of the plasma membrane (Fig. 4), consistent with the determined crystal structure of the KcsA K^+ channel. Glutamate or aspartate residues at positions 172 and 224 of Kir2 channels, present within the M2 transmembrane domain and carboxy terminal tail, respectively, have been demonstrated to play a role in their strong rectification. Consistent with this view, weak inward rectifiers Kirl and Kir3-6 family members lack these charged residues at equivalent positions, but become strong inward rectifier channels on mutating glutamate or aspartate into these analogous positions (reviewed by Cole and Clement-Chomienne, 2000).

Recently, the expression of Kir2.1 mRNA, but not mRNA encoding the other Kir2 family members Kir2.2 and Kir2.3, was detected in isolated VSM cells of the coronary, mesenteric and cerebral arteries (Bradley et al., 1999). Moreover, cloning of Kir2.1 from VSM, and its heterologous expression, yielded Kir currents with markedly similar properties to the $Ba2^+$-sensitive VSM Kir currents recorded from isolated myocytes (Bradley et al., 1999). $Ba^{\wedge+}$ demonstrates selectivity for Kir currents when utilized in the 10-50 uM range (Quayle et al., 1997). Genetic knock-out (KO) of the

Kir2.1 gene has demonstrated a lack of Kir current in myocytes isolated from cerebral arteries of KO mice, and no observable K^+-induced vasodilation of intact, pressurized KO cerebral arteries (Zaritsky et al., 2000). These results were in contrast to data from cerebral arteries of control animals that demonstrated Kir current and K^+-induced vasodilation. Therefore, the view that Kir2.1 is the dominant molecular entity responsible for V S M Kir currents is based on the following: i) similarity of Kir2 and native V S M Kir current properties, ii) sole expression of the Kir2.1 family member in vascular myocytes, and iii) lack of V S M Kir current or K^+-induced vasodilation in Kir2.1 KO mice.

1.4.4. ATP-sensitive K^+ Channels (K^TP)

ATP-sensitive K^+ channels were first observed in cardiac myocytes (Noma, 1983). They have been shown to play an important role in insulin secretion by pancreatic (3-cells, in the prevention of cardiac ischemia-reperfusion injury, and in the control of smooth muscle contractility (Quayle et al., 1997; Aguilar-Bryan et al., 1998). Physiologically, K^TP channels are inhibited by cytoplasmic ATP and, therefore, are activated under conditions of decreased cytoplasmic [ATP]; i.e. during hypoxia or metabolic inhibition (Aguilar-Bryan et al., 1998). The K^jp channels of cardiac and pancreatic (5-cells have demonstrated ATP inhibition with Kj values of 20-100 uM, consistent with the observed activation of these channels on patch excision into ATP-free solutions (Quayle et al., 1997; Seino, 1999). K^jp channels also demonstrate activation by a broad range of K^+ channel opening drugs, for example, pinacidil and cromakalim, that function by decreasing the ATP sensitivity of channels by increasing the Kj value for

ATP inhibition (Beech et al., 1993b; reviewed by Henry and Escande, 1994). All **K-ATP** channels have demonstrated the additional characteristic of inhibition by the anti-diabetic sulphonylurea drugs, such as glibenclamide (Quayle et al., 1997).

1.4.4.1. VSM K^TP channels and control of vascular tone

The characteristics of V S M K ^ J P channels have demonstrated great variability; for example, values of single channel conductance have been reported ranging from 10-280 pS (reviewed by Quayle et al., 1997). The larger conductance K ^ j p channels (>100 pS) reported in the literature may in fact be B K ^ $_a$ channels, as some studies reported a large conductance K ^ T P channel demonstrating a C a ^ $^+$ or voltage dependence (Zhang and Bolton, 1995; Cole and Clement-Chomiene, 2000). Unlike K ^ T P channels of cardiac myocytes and pancreatic P-cells, V S M K ^ T P channels have demonstrated activation by nucleoside diphosphates (NDPs) and submillimolar ATP with Kj values for ATP inhibition that are substantially higher than that reported for cardiac and pancreatic channels (Seino, 1999). These NDP-sensitive Ky^jp channels have been recorded from rat portal vein and RPV as well as from isolated myocytes of guinea-pig coronary and rat mesenteric arteries (Kajioka et al., 1991; Beech et al, 1993a, 1993b; Dart and Standen, 1993, 1995; Zhang and Bolton, 1996). This subtype of V S M K ^ j p channels was termed K-NDP °y Bolton and colleagues due to its distinct activation by NDPs. Two distinct IvATP channels with differing single channel conductances (22 and 50 pS in 60/110 mM asymmetrical K $^+$) have been demonstrated in myocytes of the rat portal vein (Zhang and Bolton, 1996; Cole et al, 2000). The 22 pS channel displayed properties consistent with

the **KNJJP** subtype of rat mesenteric artery and RPV, and the 50 pS channel a similarity to cardiac K^TP channels (Quayle et al., 1997; Aguilar-Bryan, 1998). A role for **KA_JP** channels in maintenance of VSM basal tone has been proposed in coronary, mesenteric and renal arteries, as indicated by Em depolarization and increased vascular resistance in the presence of glibenclamide (reviewed by Daut et al., 1994; Gardiner et al., 1996). However, pulmonary and cerebral arteries have not demonstrated a role for ^ATP channels in maintaining basal tone, but K^+ channel opener- and agonist-induced relaxations have been inhibited by glibenclamide in these vessels (reviewed by Quayle et al, 1997).

1.4.4.2. Regulation of VSM K^TP channels by vasoactive agonists

Whole-cell **KATP** currents of mesenteric and cerebral arteries, urinary bladder and gall bladder smooth muscle are modulated by vasoconstrictor (histamine, phenylephrine) and vasodilatory (adenosine, calcitonin gene-related peptide) agonists signaling to PKC and PKA, respectively (Standen et al, 1989; Nelson et al., 1990; Bonev and Nelson, 1993a, 1993b; Zhang et al, 1994; Quayle et al., 1994, 1995; Kleppisch and Nelson, 1995a, 1995b; Bonev and Nelson, 1996). The differential contribution of VSM K.ATP channels to regulation of basal tone in different arterial beds may, therefore, be dependent on the state of modulation of these channels by vasoactive agonists. This has been suggested in cerebral arteries as part of an autoregulatory mechanism that contributes to maintaining constant blood flow. Under conditions of decreased blood perfusion, calcitonin gene-related peptide production by neurons is increased resulting in enhanced activity of cerebral VSM K^jp channels (Hong et al., 1994; Kleppisch and

Nelson, 1995b). More recently, it was demonstrated that single K_{sjrjp} channels of RPV and rat portal vein, but not the larger conductance cardiac-like K^-pp channel of rat portal vein, are inhibited by angiotensin II and activation of PKC (Cole et al., 2000). This is consistent with the established modulation of whole-cell vascular K^jp currents of cerebral and mesenteric myocytes by agonists leading to PKC activation (Kleppisch and Nelson 1995a; Bonev and Nelson, 1996), experiments that were performed under conditions supporting the activation of vascular K^pp channels (Beech et al., 1993a; Zhang and Bolton, 1995, 1996).

1.4.4.3. Molecular basis of VSM K^TP channels.

KATP channels are formed by the co-assembly of pore-forming Kir6 family members (Kir6.1 or 6.2) with sulphonylurea receptor proteins (SUR.1, SUR2A, SUR2B) (Fig. 4), the latter being members of the ATP-binding cassette superfamily (Aguilar-Bryan et al, 1998; Seino, 1999). The molecular compositions of K^TP channels in cardiac myocytes and pancreatic (3 cells are thought to be Kir6.2/SUR2A and Kir6.2/SUR1, respectively. This is based on the comparison of native channel pharmacology and single-channel conductance with the properties of channels cloned from these tissues and heterologously expressed (Aguilar-Bryan et al., 1998; Seino, 1999).

In contrast, the molecular identity of K^jp channels in smooth muscle has been of debate, due to the varied channel characteristics described above. Kurachi and colleagues have postulated that the combination of Kir6.1/SUR2B channels are the

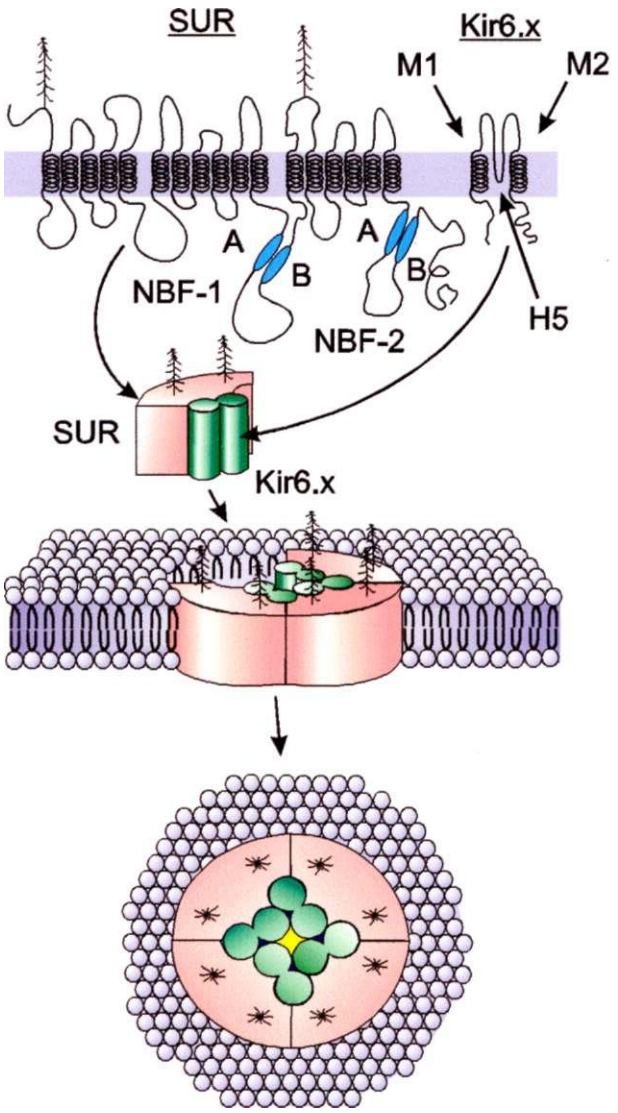


Figure 4: Proposed topology and assembly of KATP channels. KATP channels are composed of four 2-transmembrane domain, pore-forming, inward rectifier subunits of the Kir6 family and four 17-transmembrane domain sulphonylurea receptors (SUR). Kir6.X subunits contain M l , M2 transmembrane domains and a H5 domain that contributes to the K^+ selectivity filter. SURs have 2 nucleotide-binding folds (NBF), each containing a Walker A and B motif for ATP binding.

molecular correlates of VSM KNTJP channels (Yamada et al., 1997; Satoh et al, 1998), due to similarities in pharmacology, ATP activation/inhibition, NDP activation and single channel conductance (-35 pS in 145/145 mM K^+). The conductance of Kir6.1 channels is considerably smaller than that of Kir6.2 pore-forming channels (-80 pS). The differences in unitary conductance have been attributed to differences in the amino acid sequences of extracellular loops connecting the transmembrane domains with the pore-forming H5 domain of the Kir6.1 and Kir6.2 subunits (Fig. 4). Evidence for and against the heteromultimerization of Kir6.1 and Kir6.2 subunits within a functional K^jp channel complex has been provided, utilizing co-expression of the two subunits in heterologous expression systems and dominant-negative viral gene transfer technologies (Kono et al, 2000; Seharaseyon et al, 2000; Cui et al., 2001). Indeed, heteromultimerization of Kir6.1 and Kir6.2 subunits has been speculated to be a source of the reported diversity in the unitary conductance of VSM K^-pp channels (Cui et al., 2001). The dominant role of Kir6.1 in VSM, and Kir6.2 in cardiac muscle, was recently confirmed by using a Kir6.2 KO mouse (Suzuki et al, 2001). Kir6.2 KO mice lacked KATP channels in the sarcolemma of cardiac myocytes, yet no difference in VSM K^TP currents was observed in comparison to control mice. This suggests that Kir6.2 subunits constitute the pore-forming subunits of the cardiac K^jp channel, but do not make a substantial contribution to VSM K^TP channels. Furthermore, the response of VSM tissues to pharmacological K^TP manipulation in KO mice was also unchanged when compared to wild-type mice. These studies provide strong evidence for the differential

contribution of Kir6.1 and Kir6.2 pore-forming subunits to K^TP channels of vascular and cardiac myocytes, respectively.

1.5. Summary: molecular basis of VSM K^+ channels and justification of the study.

1.5.1. Kir and *BKq%* channels.

Of the four K^+ channel types implicated in the control of VSM Em and vascular tone, the molecular identity of Kir and BK^$_a$ currents is the best understood. Kir2.1 is proposed to be the dominant molecular entity underlying VSM Kir currents, due to its detected expression in VSM cells, its similar biophysical properties to native Kir currents, and lack of VSM Kir current in Kir2.1 KO mice.

BKc$_a$ pore-forming subunits assembled with the BKc$_a$f31 subunits have been proposed to form the molecular basis of VSM BK£$_a$ channels. This is supported by the selectively high level of BK^$_a$pi subunit expression in smooth muscle and a marked decrease in contribution of BK^$_a$ channels to control of vascular tone in BK(^$_a$pl KO mice.

1.5.2. Kv channels.

The molecular basis of VSM Kv channels is not established. Native VSM whole-cell Kv currents exhibit functional identity with Kvl family members of the Kv channel superfamily, in terms of their biophysical properties and 4-AP sensitivity (Table 1). These properties are not shared between native VSM Kv whole-cell currents and members of the Kv2-Kv4 families. Although, a role for Kv2-Kv4 channels in vascular

myocytes cannot be precluded, their contribution to VSM Kv whole-cell current is likely minimal. For this reason, this study investigated the expression and assembly pattern of Kv1 channel Kva and Kvp subunits in VSM.

RPV was utilized as a model system for this study due to its extensively characterized Kv currents, which demonstrate similarity to those recorded from arterial myocytes. In addition, the wealth of biophysical and functional data available on native RPV Kv currents enabled a detailed comparison of the properties of specific Kv subunits with the properties exhibited by native RPV Kv currents. The large amount of smooth muscle tissue obtainable was also well suited for the biochemical studies performed. RPV is a unique blood vessel that demonstrates spontaneous electrical activity in the form of slow membrane potential depolarizations superimposed with rapid spiking depolarizations (Hara et al., 1980). These electrical properties of RPV are unlike those of Other veins and of resistance arteries. Resistance arteries demonstrate graded changes in membrane potential that contribute to the development of myogenic tone (Nelson and Quayle, 1995). In contrast, the electrical activity of RPV is more similar to those displayed by the smooth muscles of the gastrointestinal and urogenital tracts. This may perhaps reflect the physiological function of RPV utilizing its rhythmic contractile activity to propel nutrients absorbed by the mesenteric veins of the gastrointestinal tract to the liver for metabolism.

The general hypothesis tested in this study was that, in addition to the documented expression of Kv1.5, other Kva and Kvp subunits are expressed in RPV, and that these subunits explain the functional discrepancies observed between RPV native Kv currents

and Kvl.5 homomultimeric channels. The pattern of expression of mRNA encoding Kvla and Kv(3 subunits was determined by RT-PCR, followed by detection of expressed subunit proteins with subunit-specific antibodies by Western blotting and immunocytochemistry. In addition, the assembly pattern of expressed subunit proteins into Kvl channel complexes was assessed by co-immunprecipitation. The pharmacology, with respect to 4-AP inhibition, and the regulation by PKA activation of cloned channel subunits was then compared to that of native RPV Kv current.

1.5.3. Kjyrjp channels.

Kir6.1/SUR2B channels have been proposed to underlie the VSMK^pp subtype of K^jp channels based on a number of similarities between these two channels, including: i) activation by K^+ channel openers, ii) activation by NDPs, iii) activation/inhibition by ATP, and iv) single-channel conductance. Therefore, the expression pattern of known K^-pp subunits in RPV was analyzed to provide further molecular evidence for the subunit composition of K]^j)p channels. In addition, the regulation of Kir6.1/SUR2B channels by PKC was investigated to provide functional evidence for a dominant role of Kir6.1/SUR2B subunits in forming VSM **KNDP** channels and underlying arterial whole-cell K^ **TP** currents. This was based upon the previously documented inhibitory regulation of arterial VSM whole-cell K^jp currents and single RPV VSMK^pp channels by PKC activation.

CHAPTER 2: METHODS

2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR) and molecular biology of K^+ channel subunit clones.

New Zealand white rabbits were injected in the ear artery with a lethal dose of halothane, prior to removal of the portal vein. Portal veins were dissected free of connective tissue and the endothelium was removed with a rubber policeman prior to flash freezing in liquid nitrogen. A Poly-A-Pure kit (Ambion, Austin, Texas) was utilized to extract mRNA from RPVs and freshly isolated rat or mouse brains. RT-PCR was performed with a Retroscript kit (Ambion) using SuperTaq Plus (Ambion) and subunit-specific primers. Primers were designed based on the DNA sequences of multiple species present in GenBank and aligned utilizing Genetic Computer Group software Version 9 (University of Wisconsin).

The specific primers were as follows (5'-3'), sense (S), antisense (AS):

Kir6.1	GGGACATCTATGCTTACA (S)
	GACAAGGTCAGTGGCTGAG (AS)
Kir6.2	GCCATGGTCTGGTGGCTCATCG (S)
	GAATCTGGAGAGATGCTAAAC (AS)
SUR1	TGTGCGGCACCGAGAACCAC (S)
	TCTTGTGGGCAGTCTTGATC (AS)
SUR2A	AGTTTGGACCAGTGTCACA (AS)
SUR2B	TGCACGGACAAACGAGGC (AS)

SUR2X	GCATTCTGTGGTCGAACTGGC (S)
Kvl.2	GCTAGAAGCTTATGACAGTGGCTACCGGAGA (S)
	TACGAGATCTTCAGACATCAGTTAACATTTTGG (AS)
Kvl.5	TGCGCTTTGAGACGCAGCT (S)
	GCCCAGCTCCCTCATGGA (AS)
	AAGGGGCTGCAGATCCTG (S)
	TCACAAGTCGGTTTCCCGGCT (AS)
Kvl.6	ACCTGGATATCTCAAACCTCGGTGAGCATCCT (S)
	TAACGGAATTCATGAGATCGGAGAAATCCCT (AS)
Kvp 1.1	CCGTCGAATTCATGCAAGTCTCCATAGCCTG (S)
Kvp 1.2	GCGTCGAATTCATGCATCTGTATAAACCTGCC (S)
Kvp 1.3	GATTAGAATTCATGCTGGCAGCCCGGAC (S)
Kvp2	GTCCAGAATTCATGTATCCGGAATCAACCAC (S)
Kvpl/p2	GTCCCGATATCTTA(G/T)GATCTATAGTCCTT(T/C)TTGC (AS)
Kvp3.1	CTTGAGAATTCATGCAGGTGTCTATCGCG (S)
Kvp4	GCCGAGAATTCATGTCAAGAGGGTATGGTCTG (S)
Kvp3/4	GGCACGATATCCTATTTCTTGGAATGCGATTTG (AS)

Additional Kv channel primers for Kvl.1-Kvl.4 were described previously (Epperson et al., 1999). In all experiments RT-PCR products were directly sequenced on purification from agarose gels, and obtained in at least two such experiments.

Full-length cDNA clones were prepared for Kvl.2, Kvpl.l, Kvpi.2, Kvpl.3, Kvp2.1 and Kvp2.2 via RT-PCR primers (above) designed to the ends of coding regions.

Products were then ligated into the mammalian expression vector, pcDNA3, and sequenced prior to heterologous expression experiments using HEK 293 cells. 5' and 3' imposed primer sequences including stop and start codons were then verified by 5' and 3' rapid amplification of cDNA ends (RACE). GenBank accession numbers for the rabbit Kv subunits cloned in this study are as follows: Kvl.2 AF284420; KvpM.l AF131934; Kvpl.2 AF131935; Kvpi.3 AF131936; Kvp2.1 AF247701; Kvp2.2 AF247702.

A Kvl.5-Kvl.2 tandem construct was prepared by Dr. K. Ishii (Yamagata University, Yamagata, Japan), linking the carboxy terminus of Kvl.5 to the amino terminus of Kvl.2, by the established method of Nunoki et al. (1994). Briefly, the stop codon for Kvl.5 and the start codon for Kvl.2 were removed, and a DNA sequence encoding seven glycine residues was inserted, linking the two channel coding sequences. Kir6.1 and SUR2B clones utilized in this study were of mouse origin and were obtained from Dr Y. Kurachi (Osaka University, Osaka, Japan). The rabbit ATj receptor clone was generously provided by Dr. K Burns (University of Ottawa, Ottawa, Canada). A rat Kvl.4 clone was provided by Dr K. Ishii (Yamagata University).

2.2. Antibody Production

Peptide-directed rabbit polyclonal antibodies were raised against RPV Kvp1.1, Kvp1.2, Kvpi.3, KvP2.1 and Kvp2.2 subunits. Peptides were based on the unique regions of their respective amino termini and had the following sequences:

Kvp 1.1 (CIACTEHNLKSRNGEDRLLSKQS)

Kvp 1.2 (CADIPSPKLGLPKSSESALK)

Kv|31.3 (CSGGSKDRSPKKASENVKDS)

Kvp2.1 (CYSTRYGSPKRQLQF)

Kvp2.2 (CMYPESTTGSPARLSLRQTGSPGMI)

Peptides were synthesized in the Peptide Synthesis Core Facility at the University of Calgary, conjugated to keyhole limpet hemocyanin and injected into rabbits at Macro molecular Resources at Colorado State University. Antisera were then purified by HiTrap Protein A (Pharmacia, Piscataway, NJ) followed by affinity chromatography with their respective antigenic peptides coupled via an amino terminal cysteine to Sulfolink resin (Pierce-BioLynx, Brockville, ON).

2.3. Immunocytochemistry

RPV smooth muscle myocytes were isolated using a previously described method (Aiello et al., 1995). Briefly, RPVs were dissected free of fat and connective tissue, and cut into - 4 x 4 mm squares. Samples of RPV VSM were then digested by collagenase and protease at 37 °C for 14-22 min. After digestion, the tissues were removed from the digestion solution and placed on ice for 1 h. Individual RPV vascular myocytes were then obtained by gentle trituration of the tissue samples with a fire-polished Pasteur pipette, and plated onto acid-washed glass coverslips. HEK cells were plated on plastic 2.5 cm plates and co-transfected with appropriate Kv channel clones along with a cDNA clone encoding green fluorescent protein (GFP) via Fugene 6 (Boehringer Manneheim, GmbH, Germany). Immunocytochemistry was performed on freshly isolated RPV myocytes and 24 h, post-transfected HEK cells. Cells were washed with phosphate-buffered saline

(PBS), fixed for 20 min with 3.7% formaldehyde and permeabilized for 5 min with 0.1% Triton X-100. The cells were then washed with PBS, blocked for 1 h in 1% bovine serum albumin (BSA)/PBS, and labeled overnight at 4 °C with primary antibodies in 0.5% BSA/PBS. The cells were then washed with 0.5% BSA/PBS and labeled for 1 h with anti-rabbit or anti-mouse IgG-conjugated Cy3 secondary antibodies (Jackson Labs, Bar Harbor, ME). Unbound secondary antibody was then washed out prior to visualizing immunolabeled cells. In all cases results shown are representative of results obtained in at least 2 separate experiments.

2.4. Western blotting and immunoprecipitation

HEK cells were harvested and RPV tissues were homogenized in extraction buffer containing: 20 mM Tris pH 7.5, 138 mM NaCl, 3 mM KC1, 1 mM EGTA, 2 mM EDTA, 1 mM benzamidine, 5 pg/ml aprotinin, 5 pg/ml leupeptin, 5 pg/ml pepstatin A, 1 mM phenylmethylsulphonylfluoride, 1 mM dithiothreitol and 1% Triton X-100, followed by centrifugation at 16 000 x g. Supernatant protein concentrations were determined using the DC kit (BioRad, Hercules, CA). Proteins were subjected to SDS-PAGE on 7.5-15 or 7.5-20% polyacrylamide gels prior to transfer to 0.2 pm nitrocellulose membranes. Membranes were blocked with 5% non-fat dried milk (NFDM) in 0.05% Tween-20/Tris-buffered saline (TBST) prior to labeling with primary antibodies in 1% NFDM/TBST for 2 h at room temperature. Unbound primary antibody was washed out with TBST prior to labeling with 1:10000 or 1:5000 anti-rabbit IgG-horseradish peroxidase(HRP) or anti-mouse IgG-HRP (Chemicon, Temecula, CA), respectively, in 1% NFDM-TBST for 1 h. Unbound secondary antibody was washed from membranes with TBST prior to detecting

bound antibody with the Supersignal enhanced chemiluminesence detection system (Pierce-BioLynx) and Kodak XB-1 Blue film.

For immunoprecipitation experiments, all incubations were performed while tumbling at 4 °C. RPV and HEK cell protein extracts were pre-cleared for 1 h with 25 jal of Protein A-Sepharose (Pharmacia) and incubated overnight with 5 u.g of antibody. Antibody-bound Kv channel complexes were then captured by the addition of 25 ul of Protein A-Sepharose and incubated for 2 h to facilitate binding. Protein A-Sepharose was spun down at 2000 x g, washed twice with extraction buffer, and once with Triton X-100-free extraction buffer. Immunoprecipitated channel complexes were then eluted from the beads using SDS-PAGE sample buffer prior to electrophoresis and Western blotting. In all cases results shown are representative of results obtained in at least 2 separate experiments.

2.5. Electrophysiological recordings

HEK 293 or HEK 293T cells were co-transfected with Kv channel clones or Kir6.1/SUR2B channel clones, respectively, along with a cDNA clone encoding GFP as a marker of successfully transfected cells.

2.5.1. Whole-cell and inside-out (I-O) macropatch recordings

Whole-cell current recordings and **1**-0 macropatch recordings of Kv channel clones in HEK cells were performed with an Axopatch 200A amplifier (Axon Instruments, Union City, CA) at 20-22 °C. Data were filtered at 1 kHz by an on-board 8-pole Bessel filter before 5 kHz digitization with a Digidata 1200 *AID* converter (Axon Instruments) and storage to the hard disk of a PC. Voltage clamp protocols were

performed using pClamp version 6.0 software (Axon Instruments) and data were analyzed using pClamp and Origin graphics software (Microcal Software, Northampton, MA). The bath solution for whole-cell recordings contained (in mM): 120 NaCl, 3 $NaHCO_3$, 4.2 KC1, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, and 10 Hepes (pH 7.4). The pipette solution contained (in mM): 110 K-gluconate, 30 KC1, 0.5 MgCl2, 5 Hepes, 1 GTP, 5 Na_2ATP and 10 BAPTA (pH 7.2). The pipette solution for 1-0 macropatch experiments contained (in mM): 140 KC1, 1 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose and 10 Hepes (pH 7.4) and the bath solution contained (in mM): 140 KC1, 2.5 $MgCl_2$, 5.5 glucose and 10 Hepes (pH 7.2) with, or without, 2 mM ATP and 40 nM catalytic subunit of PKA purified from bovine heart (Demaille et al., 1977). Forskolin, 8Br-cAMP and 4-aminopyridine were purchased from Sigma Chemical Co. (St. Louis, MO). Protein phosphatase 2A was purified from chicken gizzard and provided by Dr. M. Pato (University of Saskatchewan, Saskatoon, Canada). Steady-state activation curves were constructed by tail current analysis after 250 msec voltage steps between -80 and +70 mV elicited from a holding potential of -60 mV (Aiello et al, 1985).

2.5.2. Single channel recordings

Single channel currents of Kir6.1/SUR2B channels were measured by C-A and I-O patch clamp using an Axopatch 200A amplifier and Axotape 2.0 software (Axon Instruments). All experiments were performed at 20-22 °C. Bath and pipette solutions contained (in mM): 140 KC1, 2.3 $MgCl_2$, 10 glucose, 1.0 EGTA, and 10 Hepes (pH 7.4); and 140 KC1, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose, 10 Hepes (pH 7.4). For 1-0 experiments MgATP (0.5 mM) and MgADP (0.5 mM) were added to the bath. Phorbol 12, 13-

dibutyrate (PdBu), 4a-phorbol-12-13-didecanoate (PdDe), chelerythrine and glibenclamide were purchased from Sigma Chemical Co. Pinacidil and angiotensin II were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and Research Biochemicals Inc. (Natick, MA), respectively. PKC was prepared from rat brain as previously described (Allen et al., 1994) and found to be active in the presence of phosphatidylserine and diacylglycerol with no dependence on Ca^{+} for further activation. Data were filtered at 2 kHz by an on-board 8-pole Bessel filter before digitization at 10-15 kHz. Open probability was determined from amplitude histograms based on identical duration recording periods in different treatment groups of 1-3 min and 30-60 s for C-A and 1-0 patches, respectively. The open probability (Po) was expressed as NPo (number of channels (N) x mean Po of the single channels) according to the following equation: $NP_0 = (A_1 + 2A_2 + 3A_3 + ...+ nA_n)/(A_0 + A_1 + A_2 + A_3 + ...+ A_n)$, where A_0, A_1, A_2, A_3 and A_n are the areas under each histogram peak with the channels closed, one open, and simultaneous openings of 2 to n channels, respectively (Kajioka et al., 1991). An analysis of open and closed dwell times was conducted using pClamp software (Axon Instruments). A burst was defined as a train of openings of 3 or more transitions in duration.

2.5.3, Statistics

Paired Student's t test and repeated measures ANOVA followed by Student-Newman-Keuls test were used for single and multiple comparisons, respectively. Unpaired data were analyzed by Student's t test followed by a Mann-Whitney test. A level of $p<0.05$ was considered to be statistically significant.

CHAPTER 3: MOLECULAR BASIS OF VSM Kv CHANNELS

3.1. RT-PCR for Kv1a channel subunits in RPV

The expression pattern of Kv1a channel subunits in RPV was determined based on the similar biophysical characteristics and 4-AP sensitivity of these channels in comparison to native RPV Kv currents. Specific primers for Kv1a subunits (Kv1.1-1.6) and Kvβ subunits (KvP1-4) were designed, based on sequences available in GenBank, and used for RT-PCR of mRNA isolated from RPV. Brain mRNA was utilized in parallel experiments as a positive control, confirming the function of all primers employed and the integrity of each RT-PCR reaction performed. RT-PCR products from RPV mRNA were obtained for Kv1.2 (1500, 1418, 449, 367 bp, Fig. 5B), Kv1.4 (188 bp, Fig. 5D), and Kv1.5 (923, 591 bp, Fig. 5E) after 35 cycles of PCR. All products were purified from agarose gels and sequenced directly to confirm their identity. Products for Kv1.1 (710 bp, Fig. 5A), Kv1.3 (653 bp, Fig. 5C) and Kv1.6 (1590 bp, Fig. 5F) were successfully obtained utilizing brain mRNA after 35 cycles, but were consistently not detected using RPV mRNA, even with a second round of PCR (total of 70 cycles). This is consistent with a lack of expression of Kv1.1, Kv1.3 and Kv1.6 subunits in RPV VSM.

3.2. RT-PCR for Kvβ channel subunits in RPV

Since modulatory Kvβ subunits co-assemble with Kv1a family members to produce functional channel complexes, RT-PCR was performed to determine the expression pattern of these auxiliary subunits in RPV. As for Kv1a subunit RT-PCR,

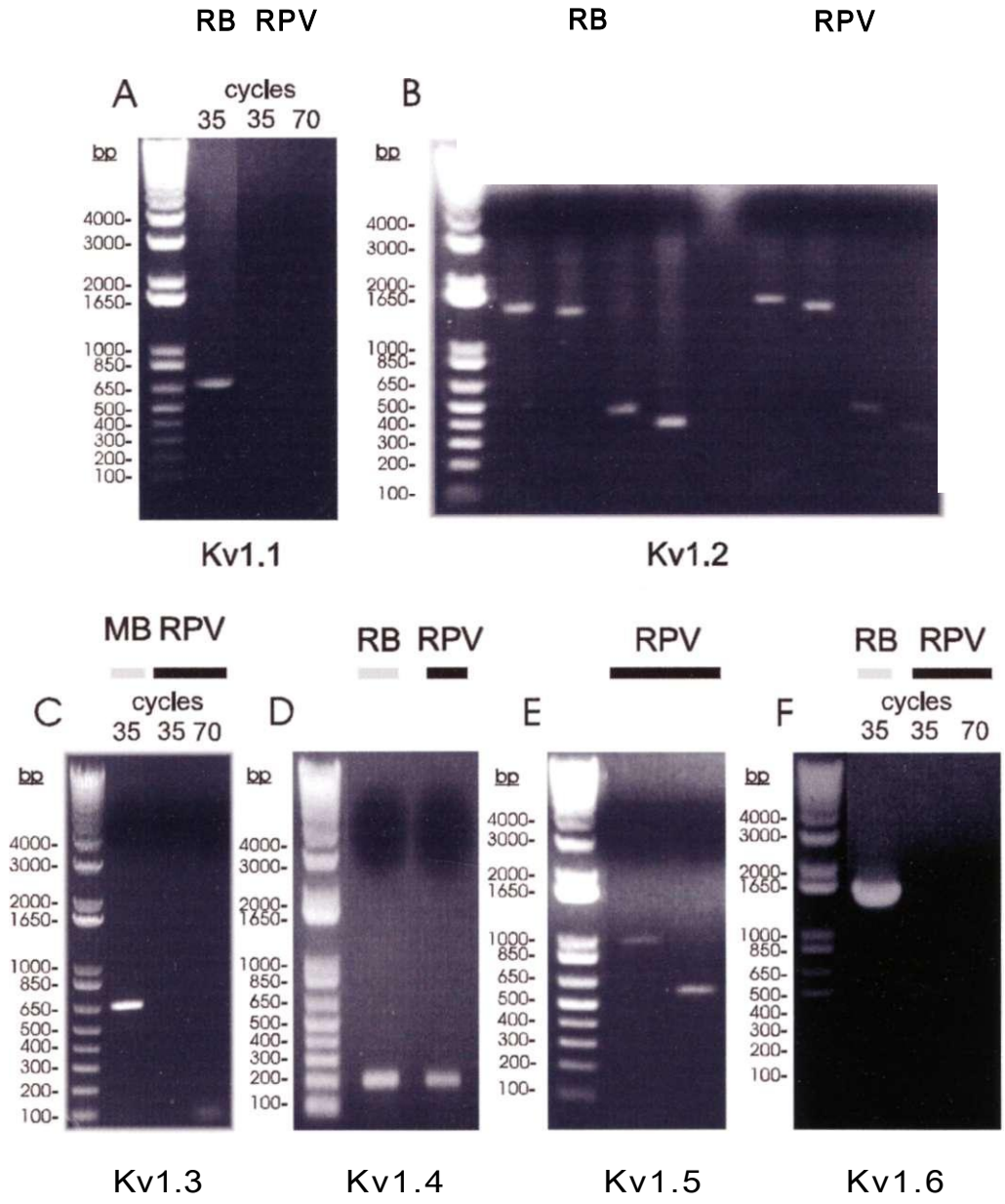


Figure 5: RT-PCR for Kv1a subunits in rabbit portal vein (RPV). A: RT-PCR for Kv1.1. 710 bp product detected in rat brain (RB), but not in RPV. **B:** RT-PCR for Kv1.2. 1500, 1418, 449 and 367 bp products detected in RB and RPV. **C:** RT-PCR for Kv1.3. 653 bp product detected in mouse brain (MB), but not in RPV. **D:** RT-PCR for Kv1.4. 188 bp product detected in RB and RPV. **E:** RT-PCR for Kv1.5. 923 and 591 bp products detected in RPV. **F:** RT-PCR for Kv1.6. 1590 bp product detected in RB, but not in RPV.

brain mRNA was utilized as a positive control alongside RPV mRNA. Products for Kvp 1.1 (1206 bp), Kvp 1.2 (1227 bp), Kvp 1.3 (1260 bp) and Kvp2 (1102, 1062 bp) were obtained from RPV (Fig. 6A). Cloning of the full-length RPV Kvp2 PCR product and subsequent screening of KvP2 clones led to the discovery of a novel KvP2 splice variant KvP2.2, subsequently reported by Akhtar et al. (1999). This splice variant lacks 14 amino acids of the Kvp2.1 N-terminus (Fig. 7), corresponding to exon 2 of the KvP2 gene (Pongs et al., 1999). RT-PCR for Kvp3.1 and Kvp4 subunits yielded 1213 bp and 750 bp products, respectively, from brain (Fig. 6B and C), but not from RPV, even after 70 cycles of PCR.

The full-length coding sequences of RPV Kvl.2, Kvpl.l, Kvpl.2, Kvpl.3, KvP2.1 and Kvp2.2 were determined utilizing 5' and 3' RACE to confirm the presence of start and stop codons. RT-PCR products encoding full-length amino acid sequences were ligated into the mammalian expression vector pcDNA3 for expression in FlEK 293 cells. The five rabbit Kvp subunit amino acid sequences demonstrated high similarity within their conserved C termini, with unique sequences in the differentially spliced regions of the N terminus (Fig. 7), consistent with Kvp subunits cloned from other species (Trimmer, 1998; Pongs et al, 1999). The amino acid sequence of rabbit Kvl.2 was markedly similar to Kvl.2 sequences cloned from 5 other mammalian species, demonstrating 99% identity with the bovine, human, mouse and rat sequences, and 98% identity with the canine sequence (Fig. 8).

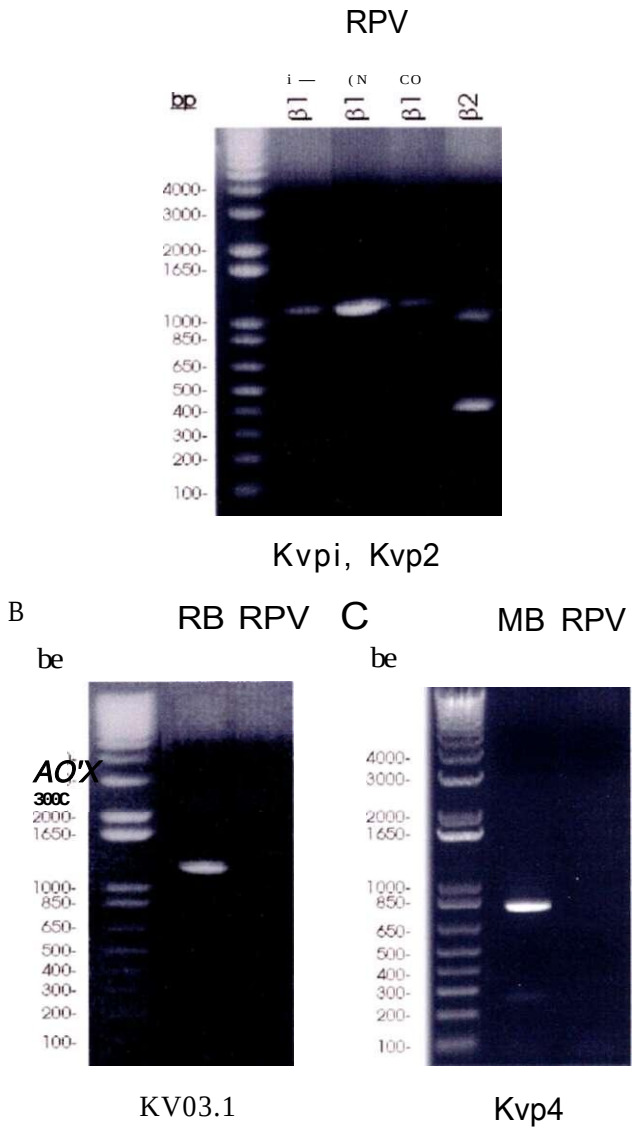


Figure 6: RT-PCR **for** Kvp **subunits in rabbit portal vein** (RPV). A: RT-PCR for Kvp 1.1 (1206 bp), Kvpl.2 (1227 bp), Kvpl.3 (1260 bp), and Kvp2 (1102 and 1062 bp). 450 bp cross reactive product in the Kvp2 lane represents a portion of the conserved region of Kvp 1 ***(arrow).*** **B:** RT-PCR for rat brain (RB) and RPV Kvp3.1. 1213 bp product detected in rat brain (RB), but not in RPV. C: RT-PCR for mouse brain (MB) and RPV Kvp4. 750 bp product detected in MB, but not in RPV.

```
                                                                    50
RPVg2.1
RPV(32.2
RPVP1.1                ___.___MQVS IACTEHNLKS RNGEDRLLSK QSSNAPNVVN
RPV31.2                MHLYKPACAD IPSPKLGLPK SSESALKCRR HLMVTKTQPQ
RPVpl.3   MLAARTGAAG SQIAEESSKL RKQAAFSGGS KDRSPKKASE NVKDSSLSPS
          51                                                   100
RPV|32.1  —MYPESTTG SPARLSLRQT GSPGMIYSTR YGSPKRQLQF YRNLGKSGLR
RPV02.2   --MYPESTTG SPARLSLRQT GSPGMJj____          YRNLGKSGLR
RPVpl.l   A.ARAKFRTV AlIARS.LGT FTPQHHISLK ESTAKQTGMK YRNLGKSGLR
RPVP1.2   A.ACWPVRPS GPTERKHLER FLCVHGVSLQ ETTKAETGMA YRNLGKSGLR
RPVP1.3   GQSQVRARQL ALLREVEMNW YLKLCELSSE HTTAYTTGMP HRNLGKSGLR
          101                                                  150
RPV32.1   VSCLGLGTWV TFGGQITDEM AEQLMTLAYD NGINLFDTAE VYAAGKAEVV
RPV32.2   VSCLGLGTWV TFGGQITDEM AEQLMTLAYD NGINLFDTAE VYAAGKAEVV
RPVPl.l   VSCLGLGTWV TFGGQISDEV AERLMTIAYE SGVNLFDTAE VYAAGKAEVI
RPVP1.2   VSCLGLGTWV TFGGQISDEV AERLMTIAYE SGVNLFDTAE VYAAGKAEVI
RPVP1.3   VSCLGLGTWV TFGGQISDEV AERLMTIAYE SGVNLFDTAE VYAAGKAEVI
          151                                                  200
RPV(32    LGNIIKKKGW RRSSLVITTK IFWGGKAETE RGLSRKHIIE GLKASLERLQ
RPV(32    LGNIIKKKGW RRSSLVITTK IFWGGKAETE RGLSRKHIIE GLKASLERLQ
RPV(31    LGSIIKKKGW RRSSLVITTK LYWGGKAETE RGLSRKHIIE GLKGSLQRLQ
RPV(31    LGSIIKKKGW RRSSLVITTK LYWGGKAETE RGLSRKHIIE GLKGSLQRLQ
RPV(31    LGSIIKKKGW RRSSLVITTK LYWGGKAETE RGLSRKHIIE GLKGSLQRLQ
          201                                                  250
RPV(52.1  LEYVDVVFAN RPDPNTPMEE TVRAMTHVIN QGMAMYWGTS RWSSMEIMEA
RPVp2.2   LEYVDVVFAN RPDPNTPMEE TVRAMTHVIN QGMAMYWGTS RWSSMEIMEA
RPVP1.1   LEYVDVVFAN RPDSNTPMEE IVRAMTHVIN QGMAMYWGTS RWSAMEIMEA
RPV31.2   LEYVDVVFAN RPDSNTPMEE IVRAMTHVIN QGMAMYWGTS RWSAMEIMEA
RPVP1.3   LEYVDVVFAN RPDSNTPMEE IVRAMTHVIN QGMAMYWGTS RWSAMEIMEA
          251                                                  300
RPVP2.1   YSVARQFNLI PPICEQAEYH MFQREKVEVQ LPELFHKIGV GAMTWSPLAC
RPVp2.2   YSVARQFNLI PPICEQAEYH MFQREKVEVQ LPELFHKIGV GAMTWSPLAC
RPV31.1   YSVARQFNMI PPVCEQAEYH LFQREKVEVQ LPELYHKIGV GAMTWSPLAC
RPVpl.2   YSVARQFNMI PPVCEQAEYH LFQREKVEVQ LPELYHKIGV GAMTWSPLAC
RPVP1.3   YSVARQFNMI PPVCEQAEYH LFQREKVEVQ LPELYHKIGV GAMTWSPLAC
          301                                                  350
RPVp2.1   GIVSGKYDSG IPPYSRASLK GYQWLKDKIL SEEGRRQQAK LKELQAIAER
RPVP2.2   GIVSGKYDSG IPPYSRASLK GYQWLKDKIL SEEGRRQQAK LKELQAIAER
RPVPl.l   GIISGKYGNG VPESSRASLK CYQWLKERIV SEEGRKQQNK LKDLSPIAER
RPVP1.2   GIISGKYGNG VPESSRASLK CYQWLKERIV SEEGRKQQNK LKDLSPIAER
RPVpl.3   GIISGKYGNG VPESSRASLK CYQWLKERIV SEEGRKQQNK LKDLSPIAER
          351                                                  400
RPVP2.1   LGCTLPQLAI AWCLRNEGVS SVLLGASNAE QLMENIGAIQ VLPKLSSSII
RPVP2.2   LGCTLPQLAI AWCLRNEGVS SVLLGASNAE QLMENIGAIQ VLPKLSSSII
RPVPl.l   LGCTLPQLAV AWCLRNEGVS SVLLGSSTPE QLIENLGAIQ VLPKMTSHVV
RPVP1.2   LGCTLPQLAV AWCLRNEGVS SVLLGSSTPE QLIENLGAIQ VLPKMTSHVV
RPVP1.3   LGCTLPQLAV AWCLRNEGVS SVLLGSSTPE QLIENLGAIQ VLPKMTSHVV
          401                420
RPVP2.1   HEIDSILGNK PYSKKDYRS*
RPVP2.2   HEIDSILGNK PYSKKDYRS*
RPVPl.l   NEIDNILRNK PYSKKDYRS*
RPVpl.2   NEIDNILRNK PYSKKDYRS*
RPVpl.3   NEIDNILRNK PYSKKDYRS*
```

Figure 7: **Amino acid sequence comparison of rabbit portal vein Kvpi.l, Kvpi.2, Kvpl.3, Kvp2.1 and Kvp2.2.** Yellow and blue highlighting represent differences in amino acid sequences, and the peptide sequences utilized to generate Kvp subunit rabbit polyclonal antibodies, respectively. GenBank accession numbers are: Kvpi.l AF131934, Kvpi.**2** AF131935, Kvp 1.3 AF131936, Kvp2.1 AF247701 and Kvp2.2 AF247702.

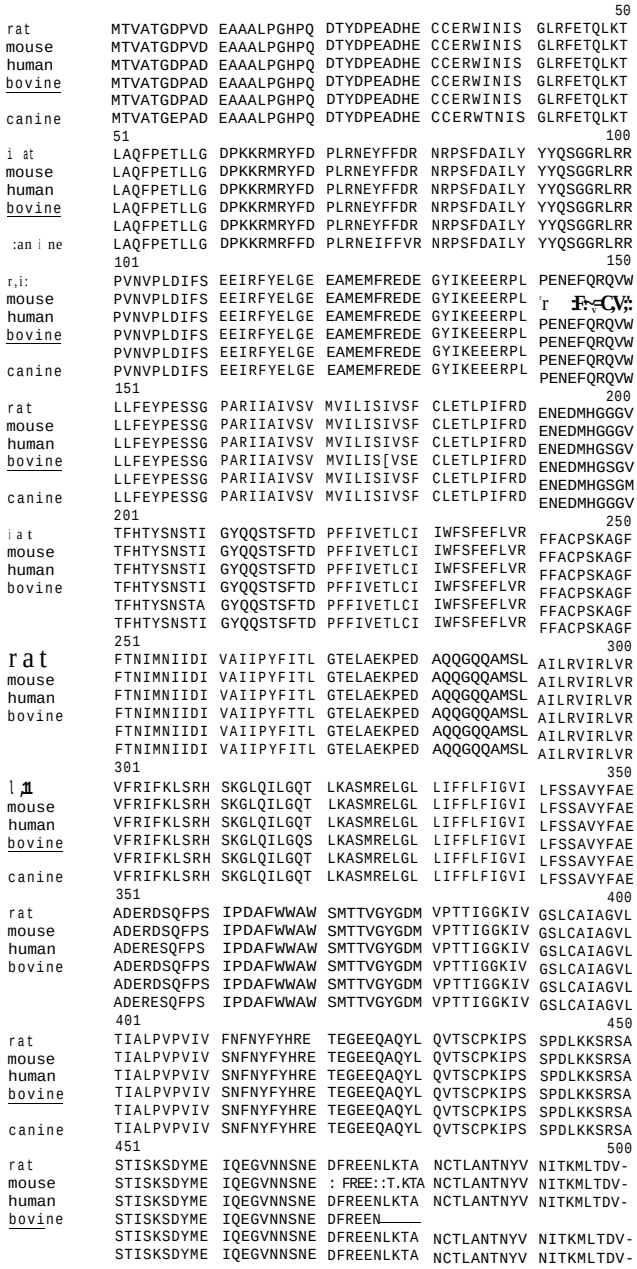


Figure 8: Comparison of the rabbit Kv1.2 amino acid sequence with Kv1.2 from five other mammalian species. Yellow highlighting represents Kv1.2 sequence differences between species; notice the marked conservation of Kv1.2 between species. GenBank accession numbers: rat M74449, mouse NM008417, human L02752, bovine X66185, rabbit AF284420 and canine LI9740.

3.3. Western blotting for RPV Kvla and p channel proteins

In order to demonstrate protein expression of Kva and Kvp channel subunits identified via RT-PCR of RPV mRNA, Kv channel subunit-specific antibodies were utilized in Western blotting (WB) experiments. Commercial antibodies to Kvl.2, Kvl.4 (Upstate Biotechnology, Lake Placid, NY) and Kvl.5 (Alomone Labs, Jerusalem, Israel) were utilized along with peptide-directed rabbit polyclonal antibodies produced to specifically recognize the splice variants of the Kvpi and Kvp2 genes. Three antibodies were raised to recognize the three splice variants of the rabbit Kvpi gene, using peptides based on the unique N-terminal domains of Kvp1.1, Kvpi.2 and Kvp1.3 (Fig. 7). Two further antibodies were raised: one specific for KvP2.1, based on the 14 amino acids present in KvP2.1, but deleted from the KvP2.2 variant, and a second KvP2 antibody, raised to the common region of the N-terminus present in Kvp2.1 and Kvp2.2, but not present in the Kvpi subunits (Fig. 7).

3.3.1. Western blotting for RPV Kvla and Kvp channel proteins in HEK cells

Antibodies to Kvla and Kvp subunits were utilized in WB experiments on protein extracts from HEK cells expressing appropriate subunit clones in order to demonstrate their specificity and functional ability. WB with Kvl.2, Kvl.4 and Kvl.5 antibodies confirmed their ability to specifically recognize Kvl.2, Kvl.4 and Kvl.5 subunits expressed in HEK cells (Fig. 9). HEK cells transfected with Kvl.2 demonstrated a single immunoreactive band calculated to be 53 kDa that was not present in mock-transfected cells; cells transfected with a different, randomly selected, Kv subunit clone

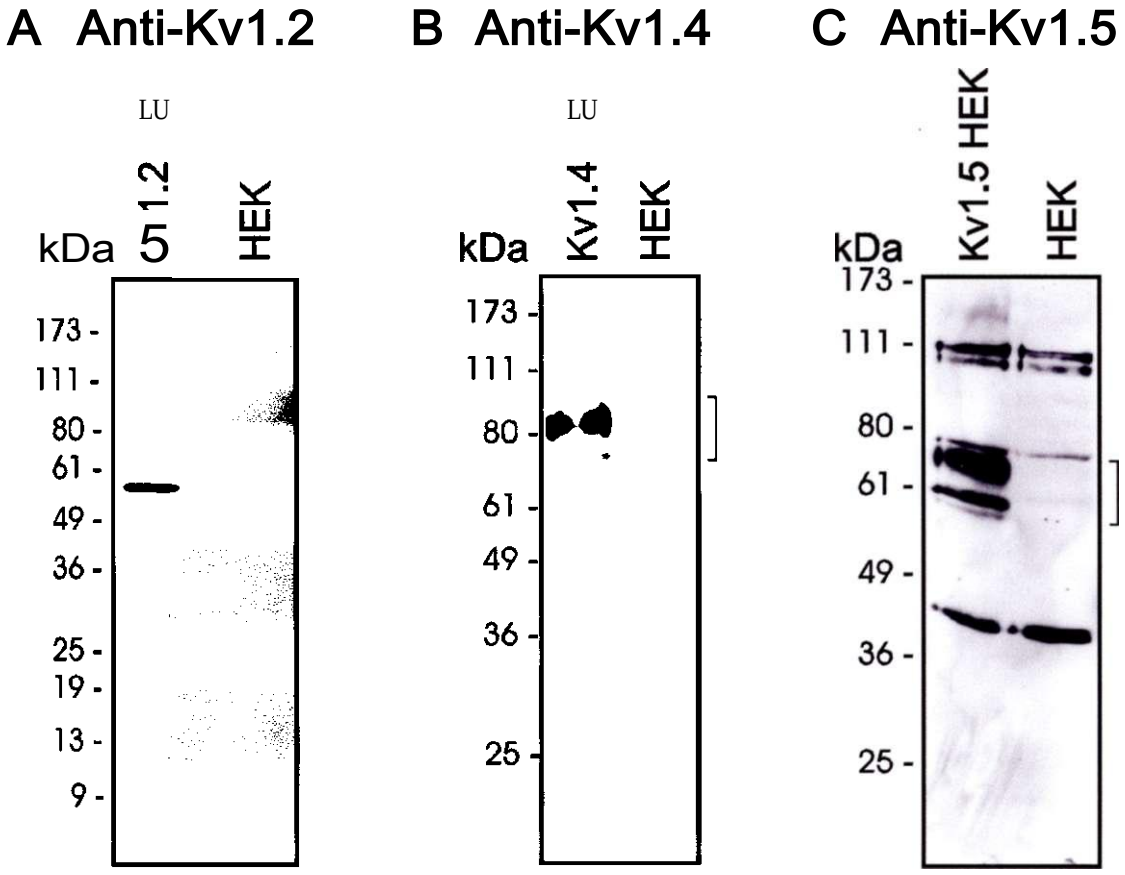


Figure 9: Western blotting for RPV Kvla subunits in HEK cells. Specific immunoreactivity demonstrated in transfected, but not in mock-transfected, HEK cells. A: Anti-Kvl.2 identified a ~55 kDa band. **B:** Anti-Kvl.4 identified a number of specific bands at ~ 80 kDa C: Anti-Kvl.5 identified a number of bands between 60 and 75 kDa.

functioned as a negative control (Fig. 9A). A number of immunoreactive bands were consistently observed for Kvl.4 (75-90 kDa) and Kvl.5 (60-75 kDa) proteins in transfected FIEK cells, which were not observed in mock-transfected cells (Fig. 9B and C, respectively). Multiple immunoreactive bands for Kvla subunits were previously demonstrated and attributed to differential glycosylation that occurs post-translationally (Shi and Trimmer, 1999).

KvpM.l, Kvpi.2 and Kv(31.3 antibodies recognized proteins of ~ 36 kDa in transfected, but not in mock-transfected, cells (Fig. 10A, B and C, respectively). Small differences in molecular weight between these Kvpi subunits were observed, as expected based on their different amino terminal sequences (Fig. 7). Anti-Kvp2 recognized a band in both Kvp2.1 and Kvp2.2 transfected cells that was smaller than the bands observed with the three Kvpi antibodies (Fig. 10D). This result was consistent with the established differences in amino acid sequence between Kvp2.1 and Kvp2.2 splice variants and the Kvpi subunits (Fig. 7). Mock-transfected cells lacked specific immunoreactivity to anti-KvP2. WB with the anti-Kvp2.1 antibody (Fig. 10E) surprisingly detected a band of ~ 33 kDa in Kvp2.1, Kvp2.2 and mock-transfected HEK cells that was consistent with the molecular weight of the band observed in Kvp2.1 transfected cells with anti-Kvp2 (Fig. 10D). Pre-absorbing anti-Kvp2.1 with the specific peptide to which the antibody was raised blocked the band present in all three HEK extracts (Fig. 10E). Furthermore, a search of GenBank revealed no other proteins with an amino acid sequence corresponding to the peptide utilized for the generation of antibodies to Kvp2.1. This, therefore, suggested the endogenous expression of Kvp2.1 protein in HEK cells. HEK

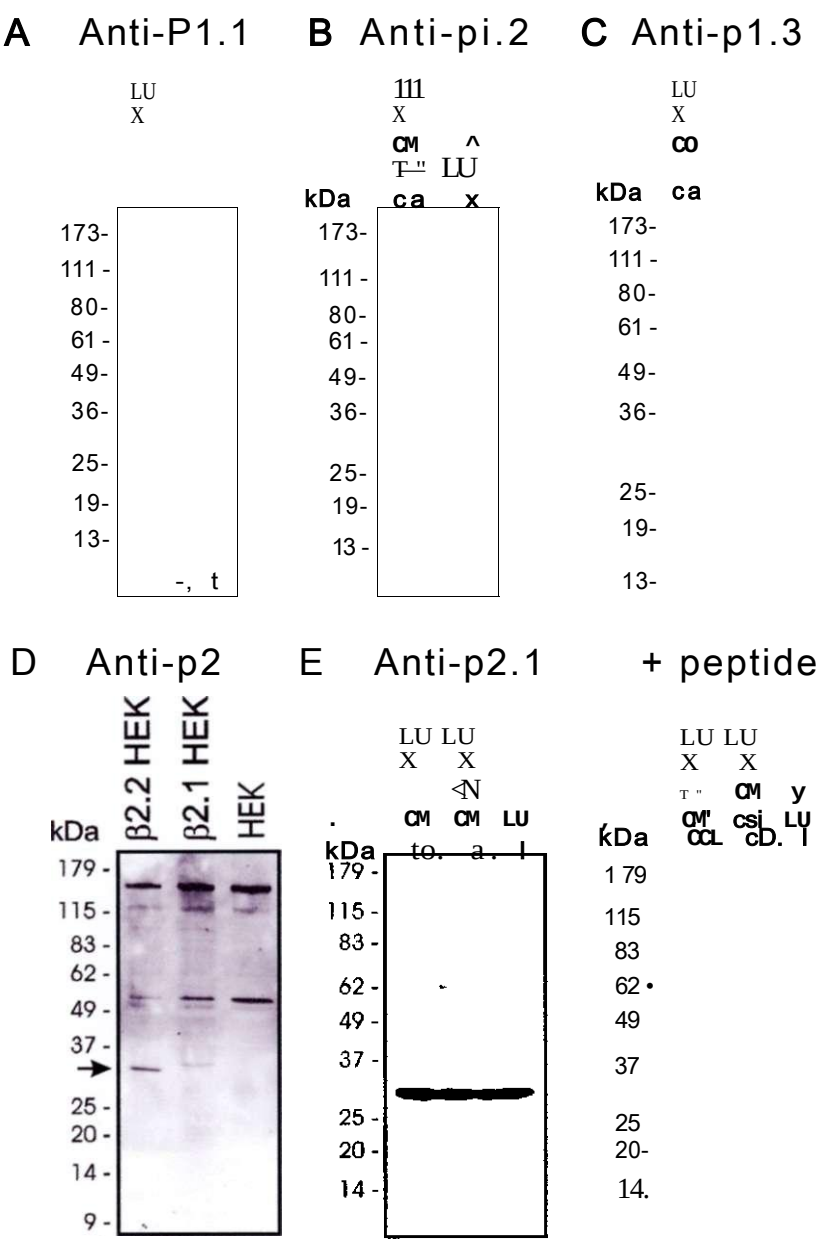


Figure 10: Western blotting for RPV Kvp **subunits in** HEK **cells.** Specific immunoreactivity demonstrated in transfected, but not mock-transfected, HEK cells following Western blotting with: A: Anti-Kvp 1.1**, B:** Anti-Kvp 1.2, **C:** Anti-Kvp 1.3, and **D:** Anti-Kvp2. E: Anti-Kvp2.1 detected Kvp2.1 in both transfected and mock-transfected HEK cells that was blocked by pre-absorption with the peptide antigen.

cell RT-PCR for Kvp2 subunits using three different sets of primers, two non-selective for the Kvp2 splice variants and a third pair spanning the spliced exon deleted from the Kvp2.2 subunit, confirmed the endogenous expression of bothKvP2.1 and Kvp2.2 splice variants in HEK cells (Fig. 11). These results contrast with the lack of Kvp subunit expression in HEK cells reported by Uebele et al. (1996) and may represent differences in passage number, or in cell culture conditions, in these two studies. Furthermore, WB of transfected HEK cell lysates detected no cross-reactivity of the novel Kvp 1.1, Kvp 1.2, Kvpi.3 and Kvp2 antibodies.

3.3.2. Western blotting for RPV Kvla and Kvp channel proteins in RPV

Following the demonstrated ability of Kvla and KvP antibodies to specifically recognize their respective subunit proteins expressed in HEK cells, these antibodies were utilized for WB of RPV proteins. WB of RPV proteins enabled the detection of Kvl.5 in RPV at a similar molecular weight to that observed following expression of RPV Kvl.5 in HEK cells (Fig. 12A). WB for Kvl.2 and Kvl.4 in RPV did not detect specific immunoreactivity to these two subunits (data not shown). However, immunoprecipitation of Kvl.2 from RPV followed by WB for Kvl.2 did detect Kvl.2 in RPV (see below), at a similar molecular weight as observed for RPV Kvl.2 expressed in HEK cells. Kvpi.2 and KvP2.1 proteins were detected by WB of RPV and demonstrated an identical molecular weight to that observed for the expressed proteins in HEK cells (Fig. 12B and C). These immunoreactive bands were absent on pre-absorbing the antibodies with their respective peptide antigens. WB with anti-Kvpi.l, anti-Kvpi.3 and anti-KvP2 did not demonstrate specific immunoreactivity in RPV protein extracts (data not shown). The

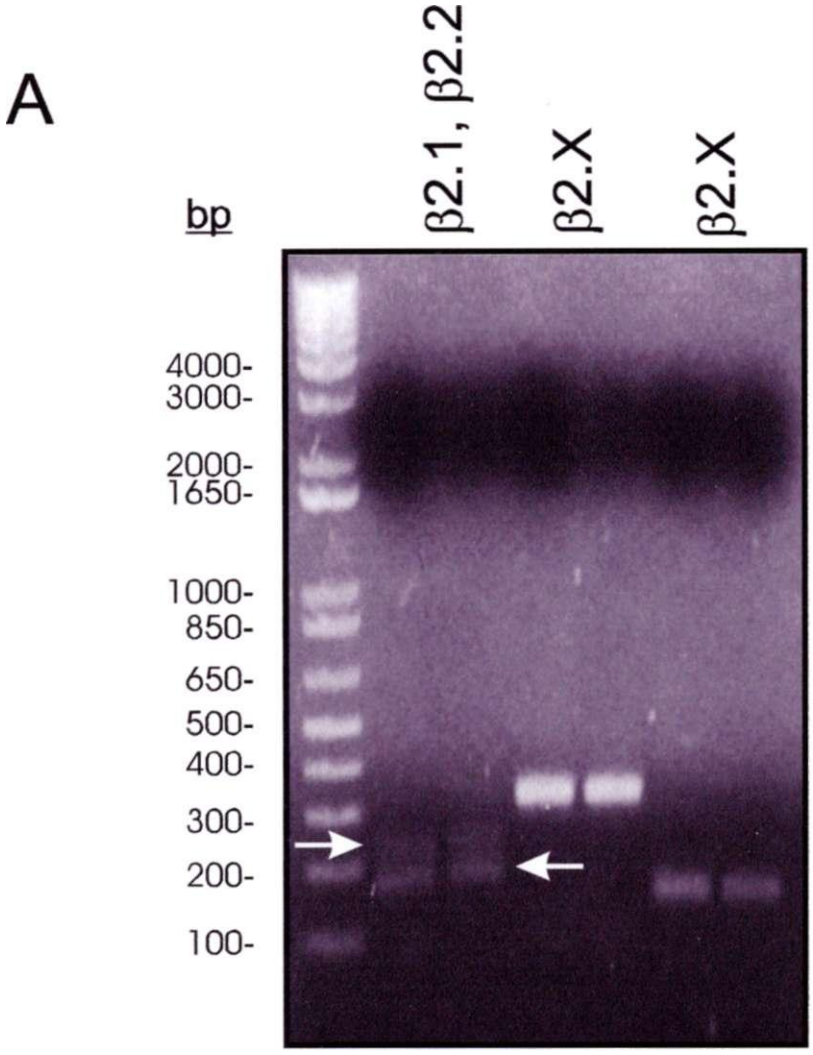


Figure 11: RT-PCR for Kvp2 subunits in HEK cells. A: Kvp2.1 and Kvp2.2 specific PCR products of 214 and 172 bp, respectively *(left lanes)*. KvP2.X PCR products of 394 *(middle lanes)* and 150 bp *(right lanes)*.

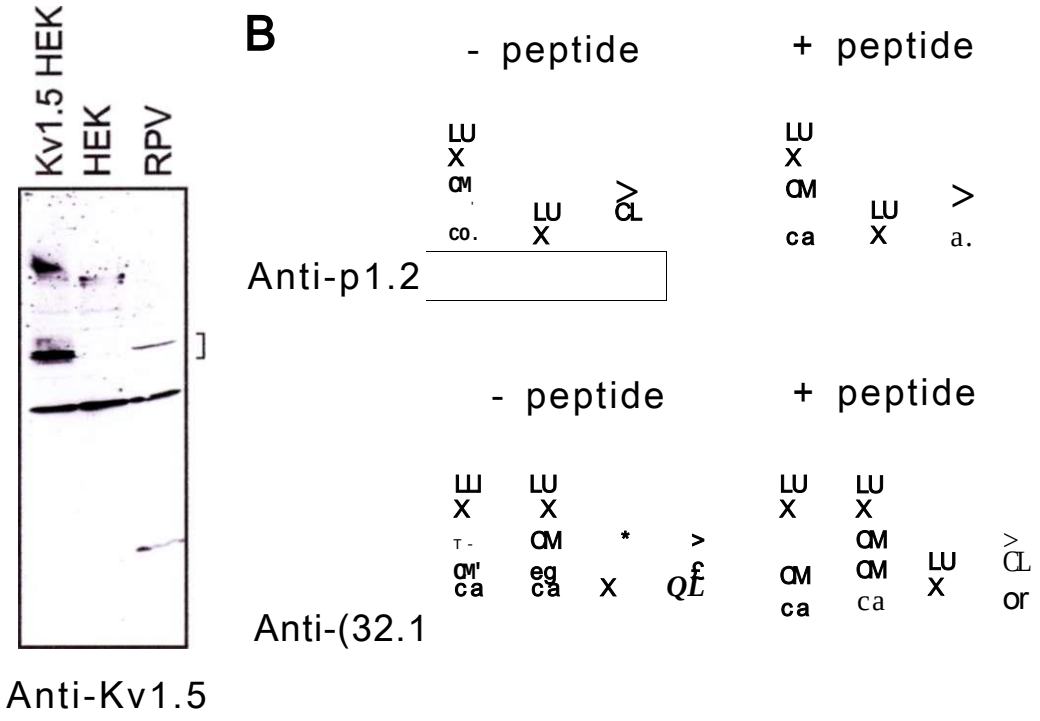


Figure 12: Western blotting for Kvl.5, Kvpl.2 and Kvp2.1 subunits in RPV. A: Anti-Kvl.5 Western blotting detected the presence of Kvl.5 in HEK cells and **RPV. B and C:** Western blotting with anti-Kvp 1.2 **(B)** and anti-Kvp2.1 (C) identified the presence of Kvp 1.2 **(B)** and Kvp2.1 (C) in transfected HEK cells and in **RPV** *(-peptide),* and a lack of immunoreactivity following pre-absorption of antibodies with the respective antigenic peptides *(+ peptide).*

lack of immunoreactivity of anti-KvpM.l, anti-Kvpi.3 and anti-Kvp2 in RPV may be due to the low titer observed for these antibodies in recognizing their respective proteins. This is demonstrated by the higher concentration of antibody and/or increased exposure times required for these antibodies on WB of transfected HEK cells (Figs. 13 and 14).

3.4. Inimunocytochemistry of RPV Kvla and Kvp channel proteins.

Previously, the expression of Kvl.5 protein in RPV was detected by immunocytochemistry using freshly isolated RPV smooth muscle cells (Clement-Chomienne et al., 1999). This provides direct evidence for the expression of Kvl.5 protein within vascular myocytes. The protein expression of the other Kv subunits detected at the mRNA level was, therefore, determined by a similar approach to demonstrate the expression of these subunits by VSM cells.

3.4.1. Immunolabelling of HEK cells expressing Kvla and Kvp channel proteins.

HEK cells expressing Kv channel subunit clones were utilized to demonstrate the specificity and functional ability of Kvl.2 and Kvl.4 monoclonal antibodies, as well as of the novel Kvp subunit antibodies, for immunocytochemistry. HEK cells were co-transfected with subunit clones and cDNA encoding green fluorescent protein (GFP) as a marker of successfully transfected cells. Kvl.2 and Kvl.4 antibodies successfully recognized the expression of Kvl.2 and Kvl.4 proteins in HEK cells, as demonstrated by the correlation of GFP fluorescence and antibody immunofluorescence, and the lack of labeling with anti-Kvl.2 and anti-Kvl.4 of Kvl.4 and Kvl.2 transfected cells,

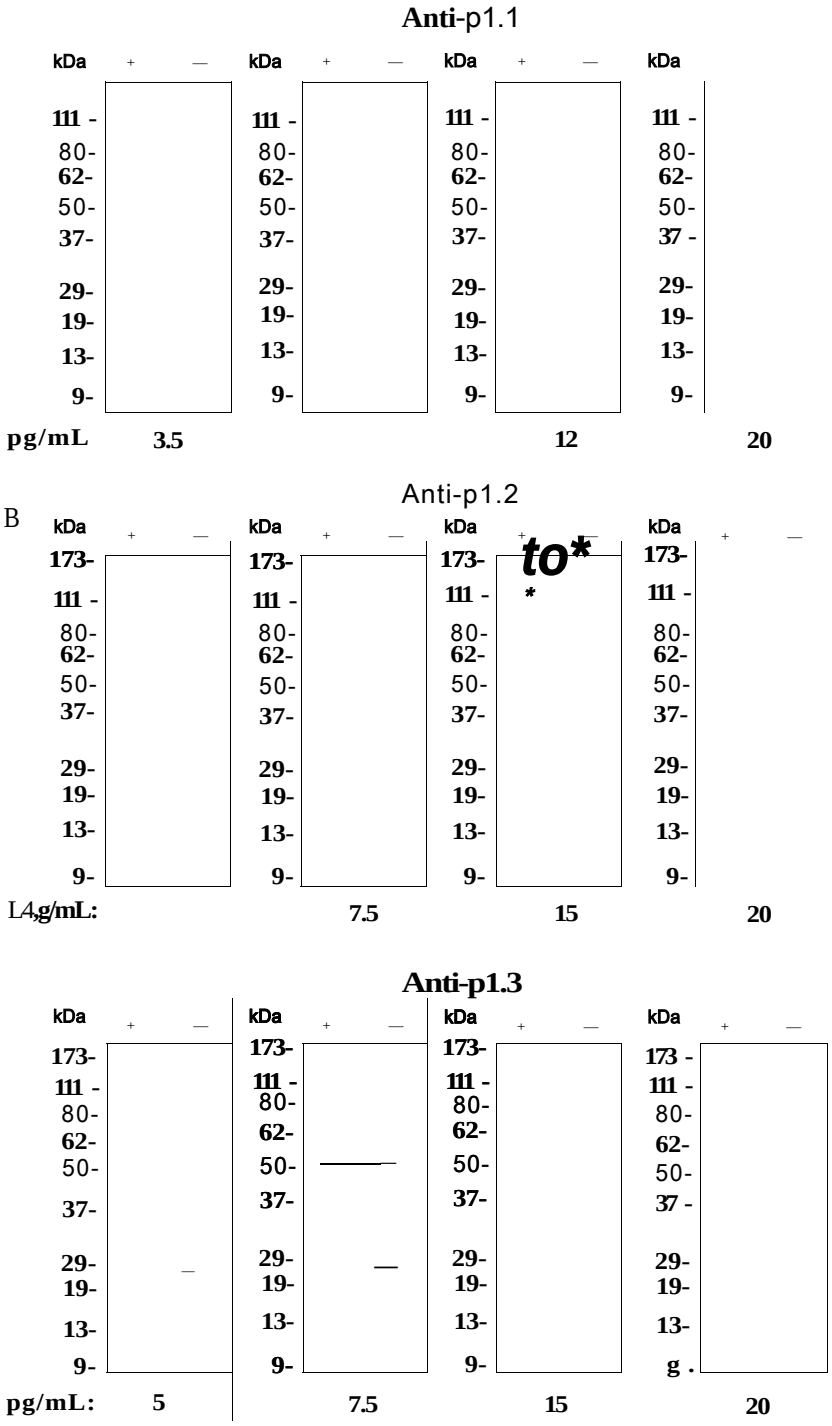
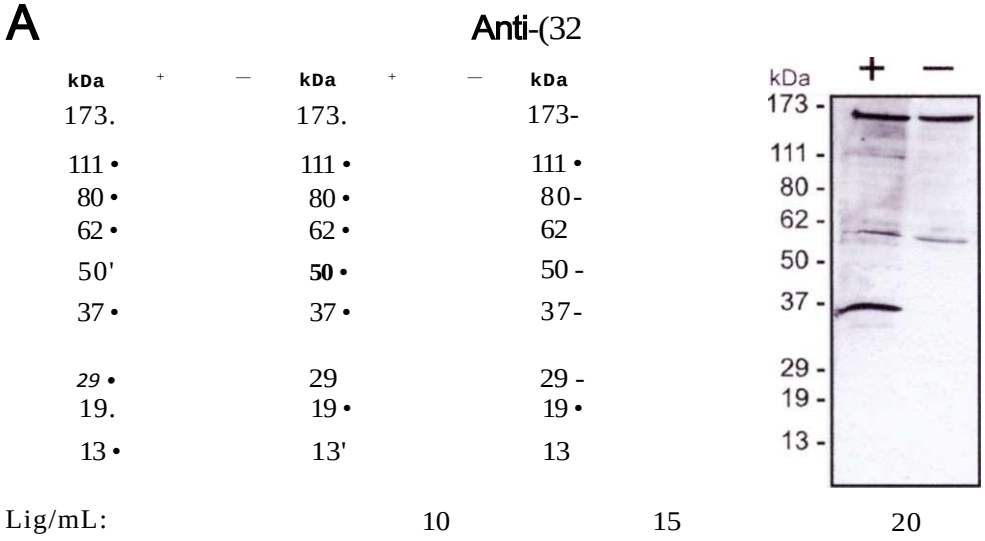


Figure 13: Titering ofanti-KvBl.l, anti-Kvpi.2 and anti-Kvpi.3 for Western blotting. Transfected (+) and mock-transfected (-) H E K cell protein extracts. A: Anti-Kvp 1.1,45 min exposure. **B:** Anti-Kvpi.2, 20 s exposure. C: Anti-Kvpi.3, 5 min exposure.



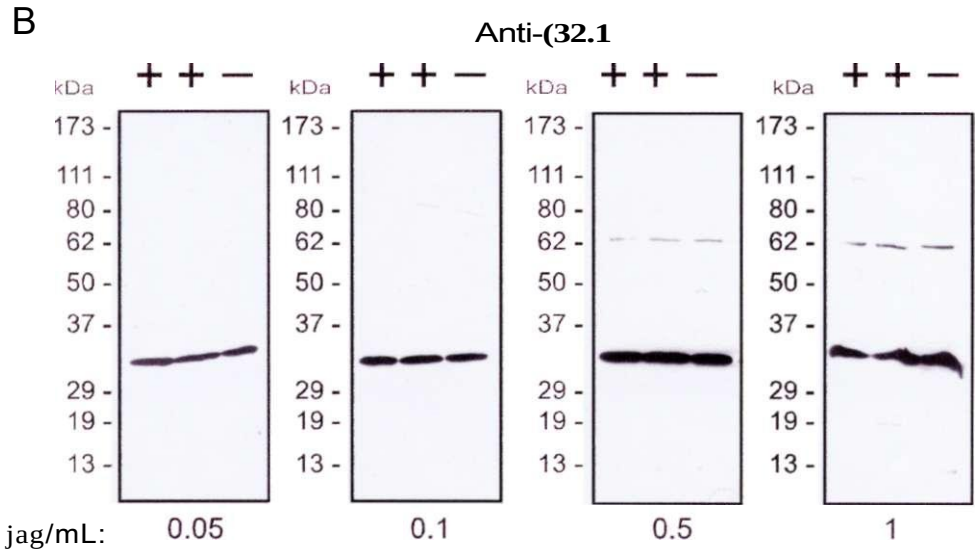


Figure 14: Titering of anti-Kvp2 and anti-Kvp2.1 for Western blotting. A: Anti-Kvp2 Western blotting of protein extracts from Kvp2.2 transfected HEK cells (+) and mock-transfected HEK cells (-), 30 min exposure. **B:** Anti-KvP2.1 Western blotting of protein extracts from Kvp2.1 or KvP2.2 transfected HEK cells (+) and mock-transfected HEK cells (-), 30 s exposure. Kvp2.1 is endogenously expressed by HEK cells.

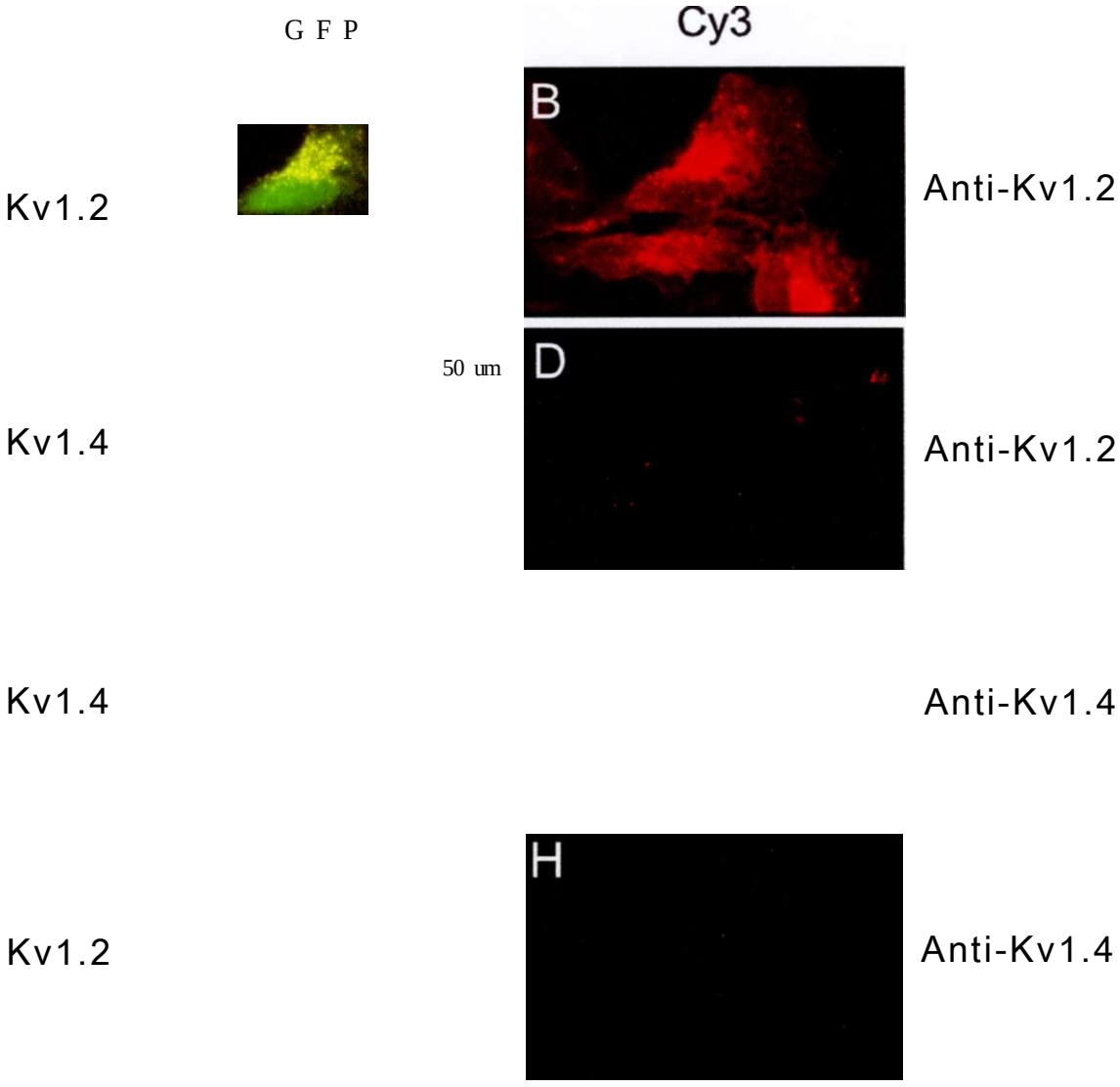


Figure 15: Immunolabeling of transfected HEK **cells for** Kvl**.2 and** Kvl**.4.** A, **B,** G, H: HEK cells transfected with GFP and Kvl.2. C, **D,** E, **F:** HEK cells transfected with GFP and Kvl.4. A, **B,** C, **D:** Cells labeled with anti-Kvl.2. E, **F,** G, H: Cells labeled with anti-Kvl.4. A, C, E, G: GFP fluorescence demonstrating successfully transfected cells with corresponding antibody labeling in **B, D, F,** H.

respectively (Fig. 15). Strong punctate immunostaining was observed for Kv1.2-expressing cells labeled with anti-Kv1.2, extending from the nuclear membrane and out to the plasma membrane. This staining likely represents a predominance of the expressed protein in the endoplasmic reticulum and/or Golgi apparatus, and is consistent with the relatively low level of Kv1.2 whole-cell current recorded from the plasma membrane of these cells and the lack of multiple glycosylated forms of Kv1.2 on WB (Fig. 9A) (Shi and Trimmer, 1999; Manganas and Trimmer, 2000).

Anti-Kvp1.2, anti-Kvp1.3, and anti-Kvp2 recognized Kvp1.2, Kvp1.3 and Kvp2.1 or Kvp2.2 protein expression in HEK cells, respectively, as demonstrated by a positive correlation between GFP fluorescence and immuno staining of transfected cells (Figs. 16 and 17). In all cases the immuno staining was blocked by pre-absorption of the antibodies with their respective antigenic peptides. Kvp1.1 and KvP2.1 antibodies were determined to be non-functional for irnmunocytochemical purposes.

3.4.2. Immunolabelling of RPV myocytes for Kv1a and Kvp proteins

Consistent with the detection of mRNA for Kv1.2 and Kv1.4 in RPV, freshly isolated RPV vascular myocytes were immunopositive for these subunits, while RPV myocytes labeled with secondary antibody alone failed to demonstrate immunofluorescence (Fig. 18). Labeling of RPV myocytes with anti-Kvp1.2 and anti-Kvpi.3 resulted in intense immuno staining that was blocked in both cases by peptide pre-absorption (Fig. 18). Anti-Kvp2 immuno staining of myocytes was not blocked by peptide pre-absorption, indicating a lack of specificity of the anti-KvP2 fluorescence in RPV

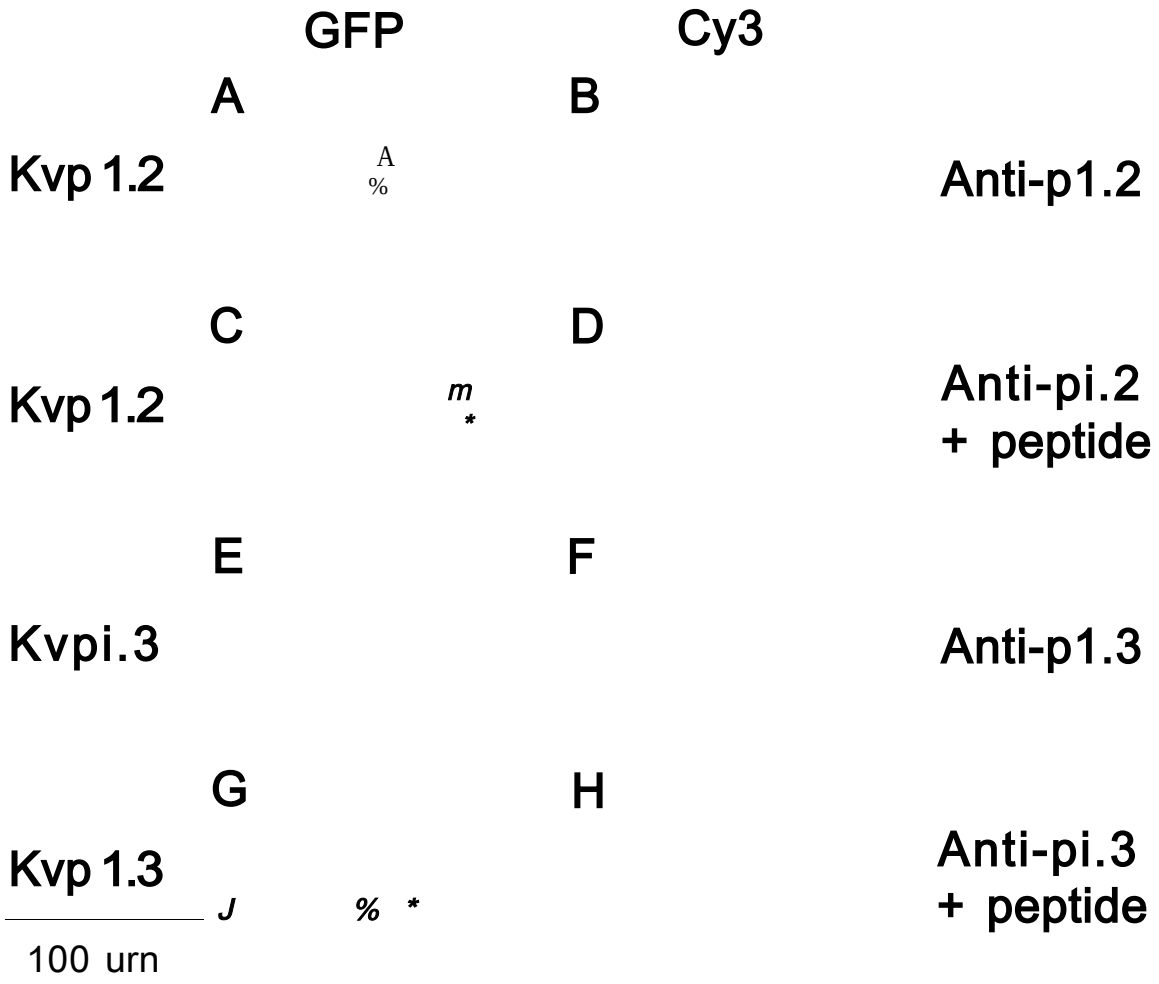


Figure 16: Immunolabeling of transfected HEK cells for Kvpi.2 and Kvpi.3. A, **B,** C, **D:** HEK cells transfected with GFP and Kvp 1.2. **E, F,** G, **H:** HEK cells transfected with GFP and Kvpl.3. **B:** Anti-Kvpl.2. **D:** Anti-Kvpi.2 pre-absorbed with antigen peptide. **F:** Anti-Kvp 1.3. **H:** Anti-Kvpi.3 pre-absorbed with antigen peptide. A, C, **E,** G: GFP fluorescence showing successfully transfected HEK cells with corresponding antibody labeling in **B, D, F, H.**

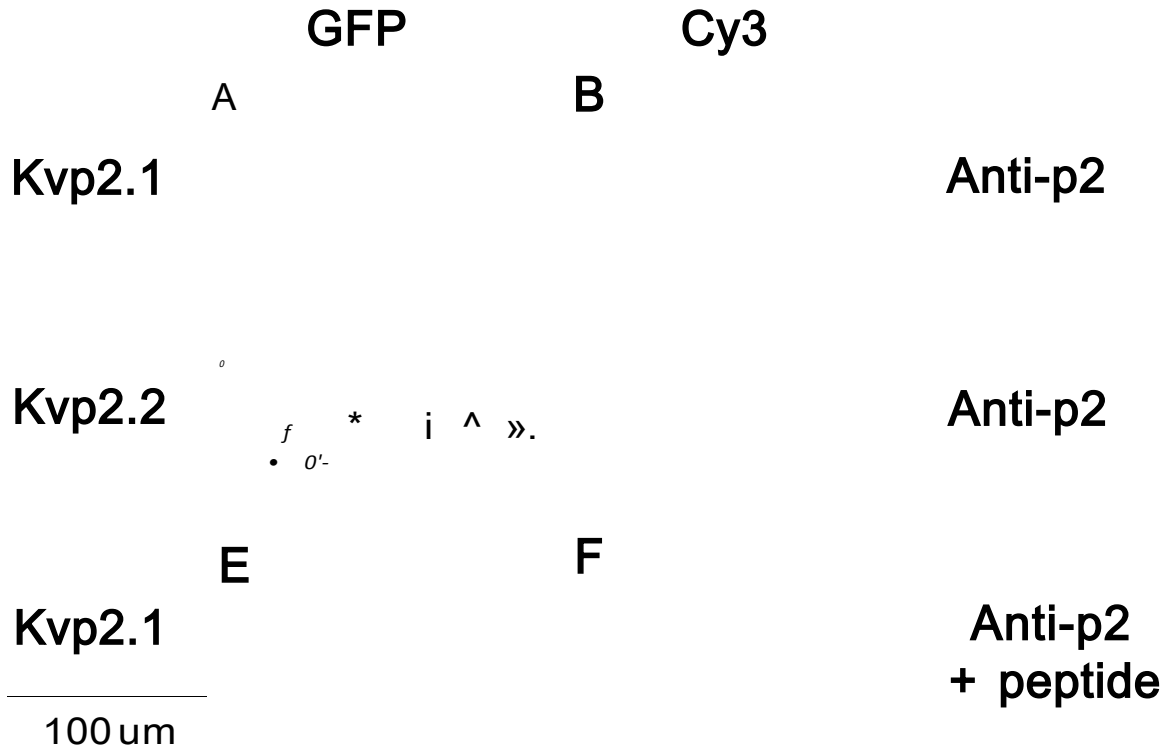


Figure 17: Immunolabeling of transfected HEK **cells for** Kvp2**.1 and** Kvp2.2. A, **B,** E, **F:** HEK cells transfected with GFP and KvP2.1. C, **D:** HEK cells transfected with GFP and Kvp2.2. **B, D:** Anti-Kvp2. **F:** Anti-Kvp2 pre-absorbed with antigen peptide. A, C, E: GFP fluorescence showing successfully transfected HEK cells with corresponding antibody labeling in **B, D, F.**

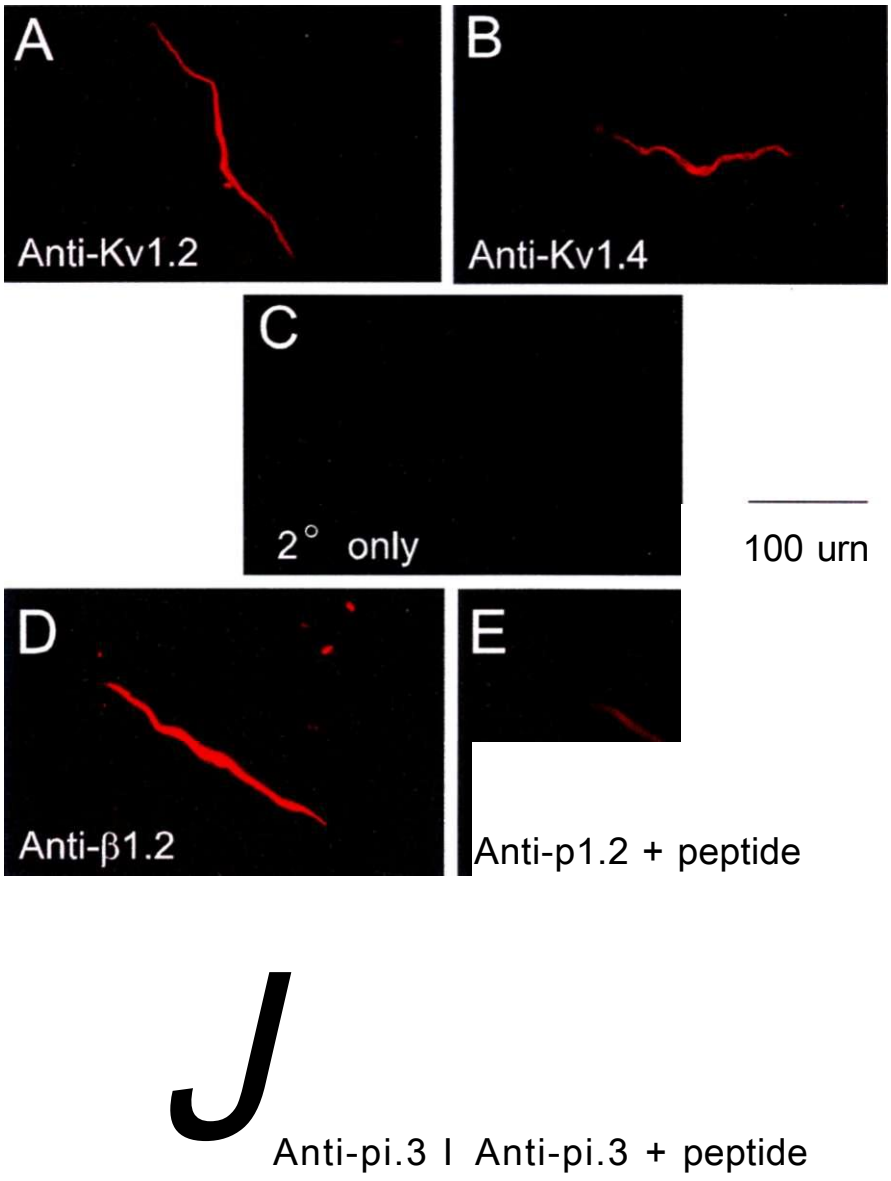


Figure 18: Immunolabeling of freshly isolated RPV vascular myocytes for Kvl a and Kv(3 subunits. A: Anti-Kvl.2. **B:** Anti-Kvl.4. C: monoclonal secondary antibody utilized in A and B alone. **D:** Anti-Kvpl.2. **E:** Anti-Kvpl.2 plus peptide. **F:** Anti-Kvp 1.3. **G:** Anti-Kvp 1.3 plus peptide. Exposure times within the following sets of pictures were equivalent: A, B and C; D and E; F and G.

myocytes. Kvp1.1 and Kvp2.1 antibodies were not utilized for immuno staining of RPV myocytes due to their demonstrated inability to immunolabel transfected HEK cells.

3.5. Immunoprecipitation of Kv channel subunits

WB and immunocytochemistry of RPV detected the expression of Kv1.2, Kv1.4, Kv1.5, Kvp1.2, Kvp1.3 and KvP2.1 subunit proteins. In order to understand the assembly of these channel subunits into functional channel complexes, immunoprecipitation (IP) experiments were performed.

3.5.1. Co-immunoprecipitation of Kv subunits expressed in HEK cells

Initial experiments utilized Kv1.2, Kv1.4, Kv1.5 and Kvpi.2 antibodies to demonstrate the ability of these antibodies to reproducibly immunoprecipitate their respective subunit proteins when expressed in HEK cells (Figs. 19 and 20). IP experiments with Kvp1.1, Kvp1.3, Kvp2.1 and Kvp2 antibodies were not successful.

The ability of Kv subunit antibodies to detect the association of Kv subunits by co-IP was also assessed in HEK cells transfected with a combination of Kv subunit clones. IP with anti-Kv1.2 of cells co-transfected with Kv1.2 and Kv1.5, or Kv1.2 and Kvp1.2, detected Kv1.5 and Kvp1.2 proteins in immunoprecipitates by WB with anti-Kv1.5 or anti-Kvp1.2, respectively (Fig. 20). IP with anti-Kv1.5 of cells co-transfected with Kv1.5 and Kv1.2, or Kv1.5 and Kvp1.2, detected Kv1.2 and Kvp1.2 in immunoprecipitates following WB with anti-Kv1.2 and anti-Kvpi.2, respectively (Figs. 19 and 20). IP with anti-Kvpi.2 of cells co-transfected with Kvp1.2 and Kv1.2, or Kvp1.2 and Kv1.5, detected Kv1.2 and Kv1.5 in immunoprecipitates following WB with

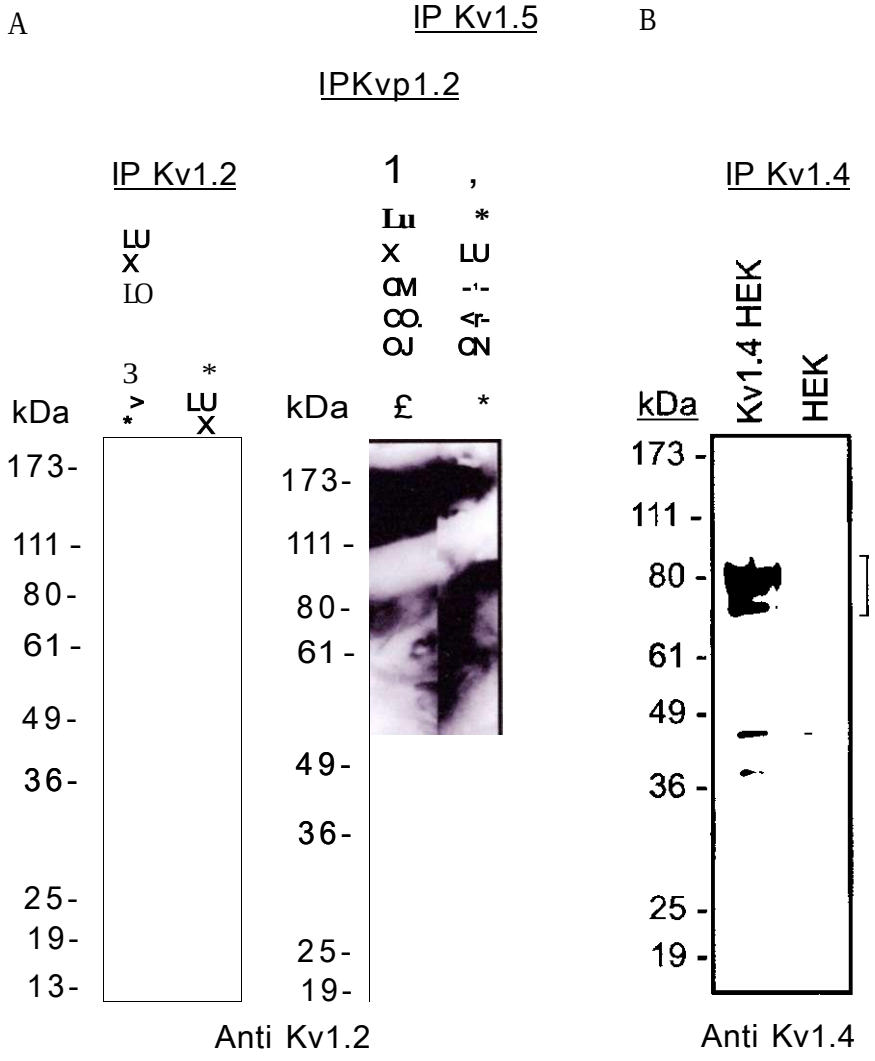


Figure 19: Immunoprecipitation of Kv **subunits from** HEK **cells.** A: Western blotting (WB) with anti-Kvl.2. Immunoprecipitation (IP) of Kvl.2 from transfected, but not mock-transfected, HEK cells *(left).* Co-IP of Kvl.2 with anti-KvpM.2 and anti-Kvl.5 on co-expression in HEK cells *(right).* **B:** WB with anti-Kvl.4. IP of Kvl.4 from transfected, but not mock-transfected, HEK cells.

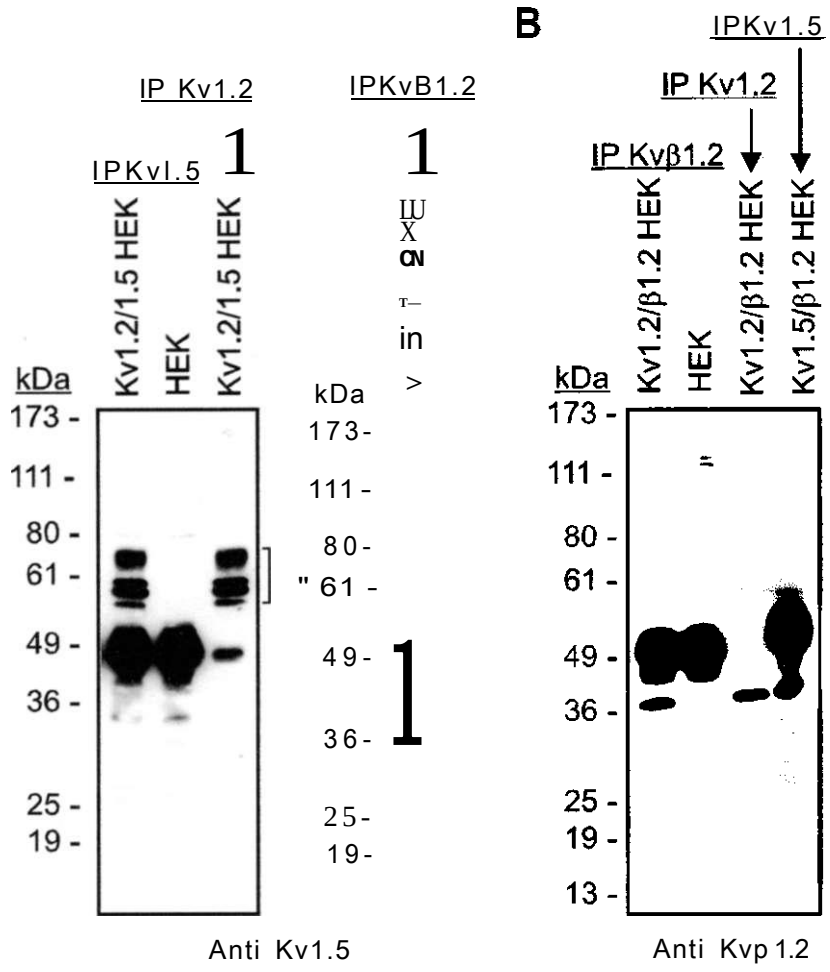


Figure 20: Immunoprecipitation of Kv subunits from HEK ceils. A: Western blotting (WB) with anti-Kvl.5. Immunoprecipitation (IP) of Kvl.5 from transfected, but not mock-transfected, HEK cells *(left).* Co-IP of Kvl.5 with anti-Kvl.2 *(left)* and anti-Kvp 1.2 *(right)* after co-expression in HEK cells. **B:** WB with anti-Kvpi.2. IP of Kvpi.2 from transfected, but not mock-transfected, HEK cells. Co-IP of Kvpi .2 with anti-Kvl .2 and anti-Kvl .5 after co-expression in HEK cells.

anti-Kvl.2 and anti-Kvl.5, respectively (Figs. 19 and 20). Co-IP of the Kvl.2 subunit with anti-Kvl.5 or anti-Kvpi.2 required considerably longer WB exposure times for detection than the reciprocal experiments, demonstrating the co-IP of Kvl.5 or Kvp1.2 subunits with anti-Kvl.2. This could not be explained by the titer of anti-Kvl.2 for WB, as ng/mL concentrations of the Kvl.2 antibody solution readily detected Kvl.2 expressed in FIEK cells and in anti-Kvl.2 immunoprecipitates of RPV.

3.5.2. Co-immunoprecipitation of Kv subunits from RPV

IP with anti-Kvl.2 from RPV protein and WB with anti-Kvl.2 yielded a band of ~ 55 kDa that was consistent with the molecular weight of Kvl.2 observed in transfected HEK cells (Fig. 21A). This band was not observed in a control RPV IP reaction using a monoclonal antibody to Kvl.6, which is not expressed in RPV. Although WB of RPV proteins for Kvl.2 did not detect Kvl.2, IP of this subunit enabled its detection, presumably due to the concentration of the subunit protein following **IP.**

IP with anti-Kvl.5 of RPV protein and WB with anti-Kvl.5 yielded a band of ~ 70 kDa (Fig. 2IB), consistent with the molecular weight of Kvl.5 detected on WBs of RPV, and of HEK cells expressing Kvl.5 (Fig. 12A). In addition, IP of RPV protein with anti-Kvl.2, or anti-Kvpi.2, followed by WB with anti-Kvl.5, detected the presence of Kvl.5 in the Kvl.2 and Kvp1.2 immunoprecipitates, thereby demonstrating the association of Kvl.2 and Kvpi.2 subunits with Kvl.5 in RPV (Fig. 21B). The immunoreactive band for Kvl.5 observed in anti-Kvl.2, anti-Kvl.5 and anti-Kvp1.2 immunoprecipitates was not present in a control IP reaction using anti-Kvl.6 (Fig. 2IB). IP with anti-Kvp1.2 and anti-Kvl.5 followed by WB with anti-Kvpi.2, detected Kvp1.2

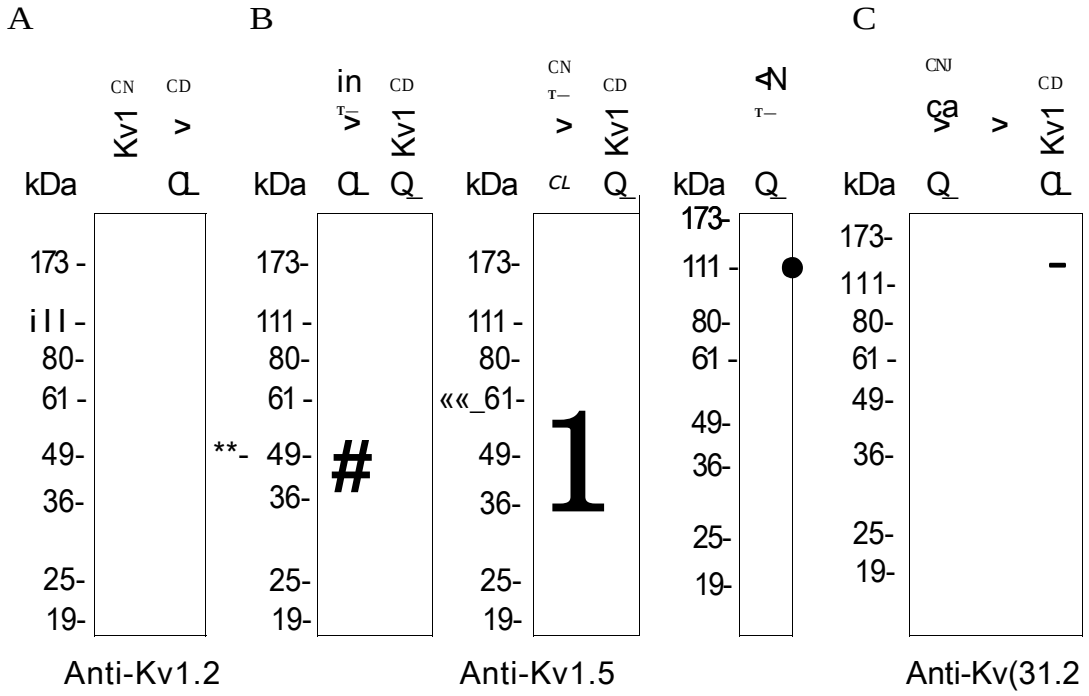


Figure 21: Immunoprecipitation of Kv subunits from RPV. A: Western blotting (WB) with anti-Kvl .2. Immunoprecipitation (IP) of Kvl .2 from RPV with anti-Kvl .2, but not with anti-Kvl.6. **B:** WB with anti-Kvl.5. IP of Kvl.5 from RPV with anti-Kvl.5 *(left),* anti-Kv|31.2 *(middle),* and anti-Kvl .2 *(right),* but not with anti-Kvl .6 *(left, middle).* C: WB with anti-Kvpl.2. IP of Kvpl.2 from RPV with anti-Kvpl.2 and anti-Kvl.5, but not with anti-Kvl.6.

in both immunoprecipitates (Fig. 21C). These data provide direct evidence for heteromultimeric assembly of Kvl.2, Kvl.5 and KvP1.2 subunits in Kv channels of VSM.

3.6. Biophysical properties of Kvl.2 and Kvl.5 channels.

Whole-cell current recordings were performed following expression of RPV Kvl.2 and Kvl.5 channel clones in HEK cells. For Kvl.5 homomultimeric whole-cell recordings, a HEK cell line stably expressing Kvl.5 was utilized. The stable cell line demonstrated steady-state activation of Kvl.5 current with a half-maximal availability (Vrj.5) of -13.5 ± 1.2 mV and a slope factor *(k)* of 11.1 ± 1.1 mV (n=15) (Table 2). These values were different from those obtained previously following the transient expression of Kvl.5 in the same cell line, which demonstrated Kvl.5 current with a **VQ**.5 of -5.3 ± 1.7 mV and a *k* value of 9.1 ± 0.7 mV (Clement-Chomienne et al., 1999). Whole-cell currents due to Kvl.2 homomultimeric channels were recorded following the transient expression of Kvl.2 in HEK cells demonstrating steady state activation of Kvl.2 current with a Vo **5** of -1.9 ± 0.7 mV and a *k* value of 15.9 ± 0.7 mV (n=6), similar to values reported for Kvl.2 expressed in Xenopus oocytes **VQ**.5 of -1.66 ± 0.30 mV and a *k* value of 7.25 + 0.25 mV (Table 2; Accili et al, 1997b). Co-expression of Kvl.2 and Kvl.5 in HEK cells yielded whole-cell currents with a **VQ**.5 of -18.1 ± 0.7 mV and a *k* value of 9.1 ± 0.7 mV (n=4) for steady-state activation (Table 2). These values were consistent with the steady-state activation values obtained from the expression of a tandem construct prepared by linking the carboxy terminus of Kvl.5 to the amino terminus of Kvl.2 by a

Table 2: Steady-state activation and inactivation properties of RPV VSM Kdr and heterologously expressed channels composed of Kv1.2 and Kv1.5 subunits.

	Activation $V_{0.5}$ (mV) k (mV)	**Inactivation** $V_{0.5}$ (mV) k (mV)	**Reference**
RPV Kdr	-16.1 ± 3.1 8.0 ± 1.3 (n=6)	-36.4 ± 1.5 7.0 ± 0.8 (n=5)	Clement-Chomienne et al, 1999
Kv1.2 HEK	-1.9 ± 0.7 15.9 ± 0.7 (n=6)	ND	Present study
Kv1.2 Xenopus oocytes	-1.6 ± 0.3 7.2 ± 0.3 (n=7)	-15* -7.7* (n=8)	Accili et al., 1997b *Hart et al., 1993
Kv1.5 HEK	-5.3 ± 1.7 9.1 ± 0.7 (n=7)	-21.2 ± 5.5 5.5 ± 0.4 (n=4)	Clement-Chomienne et al., 1999
Kv1.5 stable HEK	-13.5 ± 1.2 11.1 ± 1.1 (n=15)	ND	Present study
Kv1.2/Kv1.5 HEK	-18.1 ± 0.7 9.1 ± 0.7 (n=4)	ND	Present study
Kv1.2/Kv1.5 Xenopus oocytes	ND	-13 7.3 (n=6)	Russell et al., 1994
Kv1.5-Kv1.2 tandem HEK	-15.1 ± 0.9 11.3 ± 0.9 (n=5)	ND	Present study

ND = not determined

polyglycine linker that demonstrated a **VQ 5** of -15.1 ± 0.9 mV and a *k* value of 11.3 ± 0.9 mV (n=5) (Table 2).

3.7. **Pharmacology of native VSM RPV Kdr and channels due to Kv1.2 and Kv1.5 subunits.**

The assembly of Kv1.2/Kv1.5 heteromultimeric channels in RPV, as demonstrated by co-immunoprecipitation experiments, is consistent with the similar biophysical properties shared by RPV Kdr and expressed Kv1.2/Kv1.5 heteromultimers, and dissimilarity of RPV Kdr and Kv1.2 homomultimers (Table 2). To provide further evidence for Kv1.2/Kv1.5 channels in RPV VSM the pharmacology of native RPV Kdr current and expressed channels due to Kv1.2 and/or Kv1.5 subunits was investigated.

3.7**.1. Pharmacology of RPV Kdr current**

The 4-AP sensitivity of RPV Kdr is consistent with that of Kv1 channels, with an **IC50** value of **~200 11M** (Clement-Chomienne et al., 1999; Kerr et al., 2001), but not with that of Kv2-4 channels (Kirsch and Drewe, 1993; Grissmer et al, 1994; Kanemasa et al, 1995; Fiset et al, 1997; Schmalz et al., 1998). RPV Kdr also demonstrates a characteristic, positive shift in steady-state activation in the presence of 4-AP; for example, in the presence of 1 mM 4-AP, **VQ**.5 shifted from -14.1 ± 2.2 mV to +5.3 ± 2.2 mV in the presence of 4-AP (Kerr et al., 2001). This is consistent with reports of the effects of 4-AP on the steady-state activation of coronary and mesenteric VSM Kdr (Remillard and Leblanc, 1996; Plane and Cole, 2000; Lu et al., 2001), and with the effects of 4-AP on the gating currents of non-conducting mutant *Shaker* channels (Loboda and Armstrong, 2001). It is well established that charybdotoxin, isolated from

scorpion venom, is a selective blocker of homomultimeric Kv1.2 channels of the Kv family, whereas heteromultimeric channels containing Kv1.2 and other Kv1a subunits, such as Kv1.5, are unaffected by the toxin at <100 nM (Russell et al., 1994). Significantly, RPV Kdr demonstrates a lack of sensitivity to 50 nM charybdotoxin, indicating that Kv1.2 homomultimeric channels do not contribute to RPV Kdr current (Kerr et al., 2001).

3.7.2. Pharmacology of Kv1.2 and Kv1.5 channel subunits

The effect of 4-AP on Kv1.2 and Kv1.5 whole-cell currents was assessed following the expression of these subunits in HEK cells. Block by 1 mM 4-AP was associated with a positive shift in the steady-state activation of Kv1.2, but not Kv1.5, homomultimeric channels (Fig. 22B and A, respectively). Co-expression of Kv1.2 and Kv1.5, or expression of a Kv1.5-Kv1.2 tandem construct that results in a known 1:1 ratio of Kv1.5;Kv1.2 subunits in channel complexes, resulted in currents that demonstrated a positive shift in steady-state activation in the presence of 4-AP (Fig. 22C and D). The lack of Kv1.2 homomultimers in RPV, as demonstrated by the use of charybdotoxin, in combination with a lack of a shift in steady-state activation of Kv1.5 homomultimeric channels in the presence of 4-AP, provides strong evidence that heteromultimers, but not homomultimers of Kv1.2 and Kv1.5 subunits are the major determinant of 4-AP-sensitive Kdr current of RPV.

3.8. Regulation of cloned RPV Kv channel subunits by PKA

VSM Kdr channels of RPV have demonstrated modulation by (3-adrenoceptors and PKA activation (Aiello et al., 1995, 1998). For this reason, the modulation of Kv1.2 and Kv1.5 channels by PKA activation was assessed upon expression of these channel

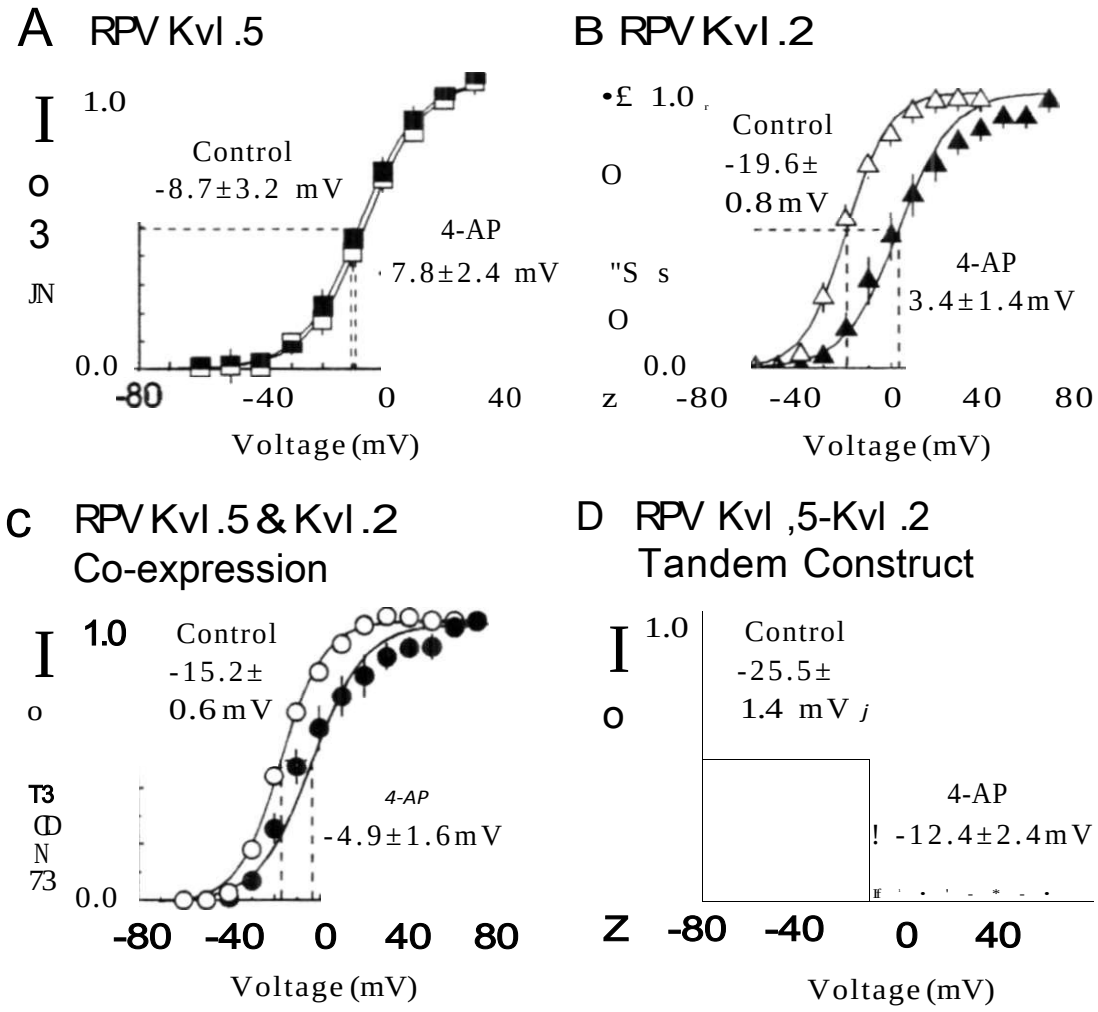


Figure 22: Effect of 1 mM 4-AP on steady-state activation of Kvl.2 and Kvl.5 channels. A: 4-AP treatment does not affect the steady-state activation of homomultimeric Kvl.5 channels. **B:** 4-AP treatment shifts the steady-state activation of homomultimeric Kvl.2 channels to more positive voltages. C, **D:** 4-AP treatment shifts the steady-state activation of heteromultimeric Kvl.2/Kvl.5 channels, produced by the co-expression of Kvl.2 and Kvl.5 subunits, or by the expression of a Kvl.5-Kvl.2 tandem construct, respectively. Respective $V_{0.5}$ values for each steady-state activation curve are indicated *(open symbols - control, closed symbols -1 mM 4-AP).*

clones in HEK cells in an attempt to understand the molecular basis of PKA modulation of the native Kdr current.

3.8.1. Kv1.5 homomultimeric channels

Whole-cell currents from the HEK cell line stably expressing RPV Kv1.5 channels were recorded. Application of the adenylyl cyclase activator, forskolin (1 uM), or the cAMP analogue 8-Br-cAMP (500 uM) to activate PKA had no effect on whole-cell Kv1.5 current amplitude after 10 min of treatment (Fig. 23). This result indicates a lack of modulation of Kv1.5 by PKA activation.

The expression level of Kv1.5 was very high in the stable cell line, as indicated by the large whole-cell currents recorded. Therefore, it was questioned whether sufficient endogenous PKA was present in the cells and/or located in the appropriate cellular location, to modulate channels following activation by forskolin or 8Br-cAMP. To address this issue, two sets of experiments utilizing the purified, constitutively active, catalytic subunit of PKA (csub-PKA) were performed. First, 1-0 membrane patches excised from Kv1.5 stable cells were utilized applying csub-PKA directly to the cytoplasmic face of Kv1.5 channels via inclusion in the bathing solution. It was not possible to observe single-channel activity in these 1-0 patches due to the high level of Kv1.5 expression in the cell line. For this reason, macropatch currents, similar to whole-cell currents, were recorded under conditions of symmetrical 140/140 (mM) K^+, as shown in figure 24. Based on the established single-channel conductance of RPV Kv1.5 of 18 pS (Clement-Chomienne et al., 1999), it was estimated that up to 2000 Kv1.5 channels were present in the patches utilized for these experiments. Consistent with the

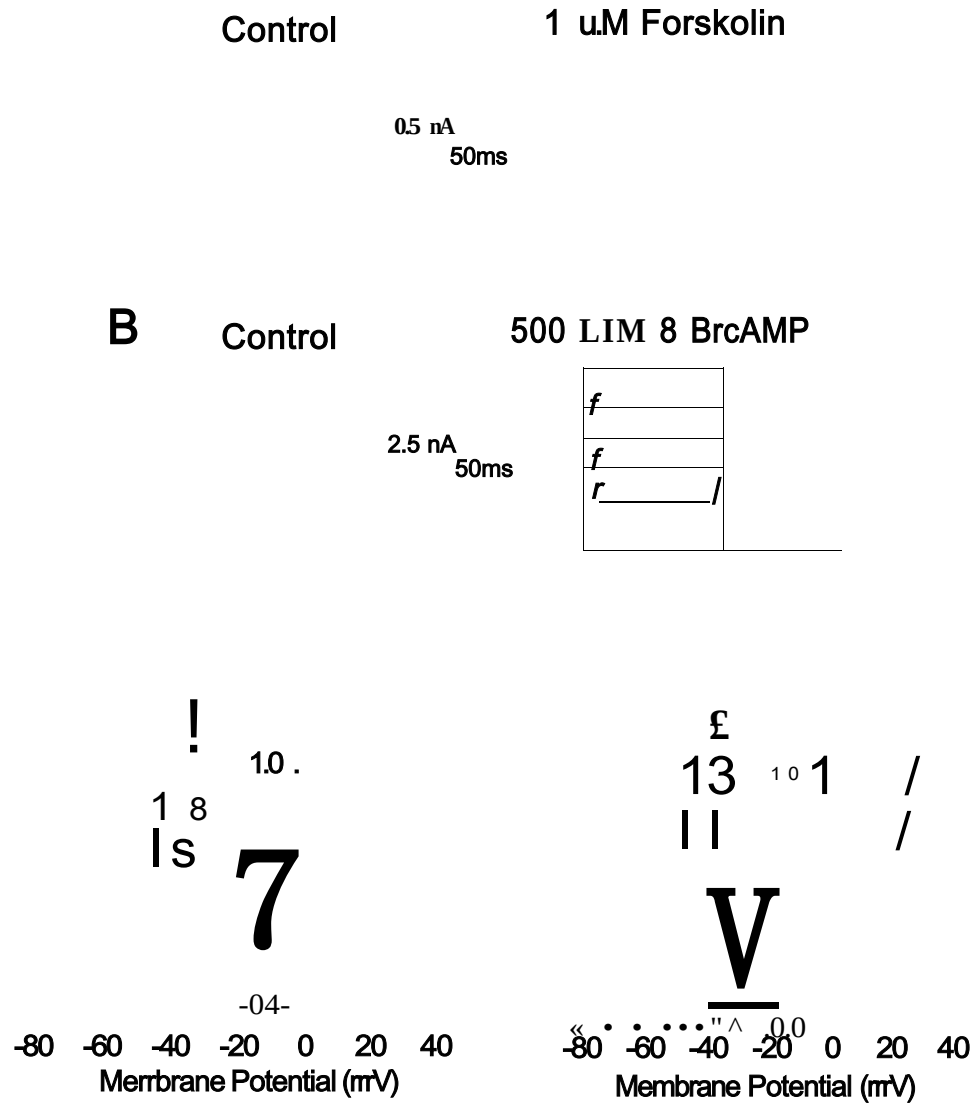


Figure 23: Effect of PKA activation by forskolin or 8Br-cAMP on Kvl.5 whole-cell current. A, B: Representative recordings showing a lack of effect of 1 u.M forskolin and 500 pM 8Br-cAMP, respectively, on Kvl.5 whole-cell currents. C, D: Normalized average I-V relations for 3 cells exposed to 1 pM forkolin and 500 pM 8Br-cAMP, respectively *(open circles-control, closed circles-forskolin / 8Br-cAMP).*

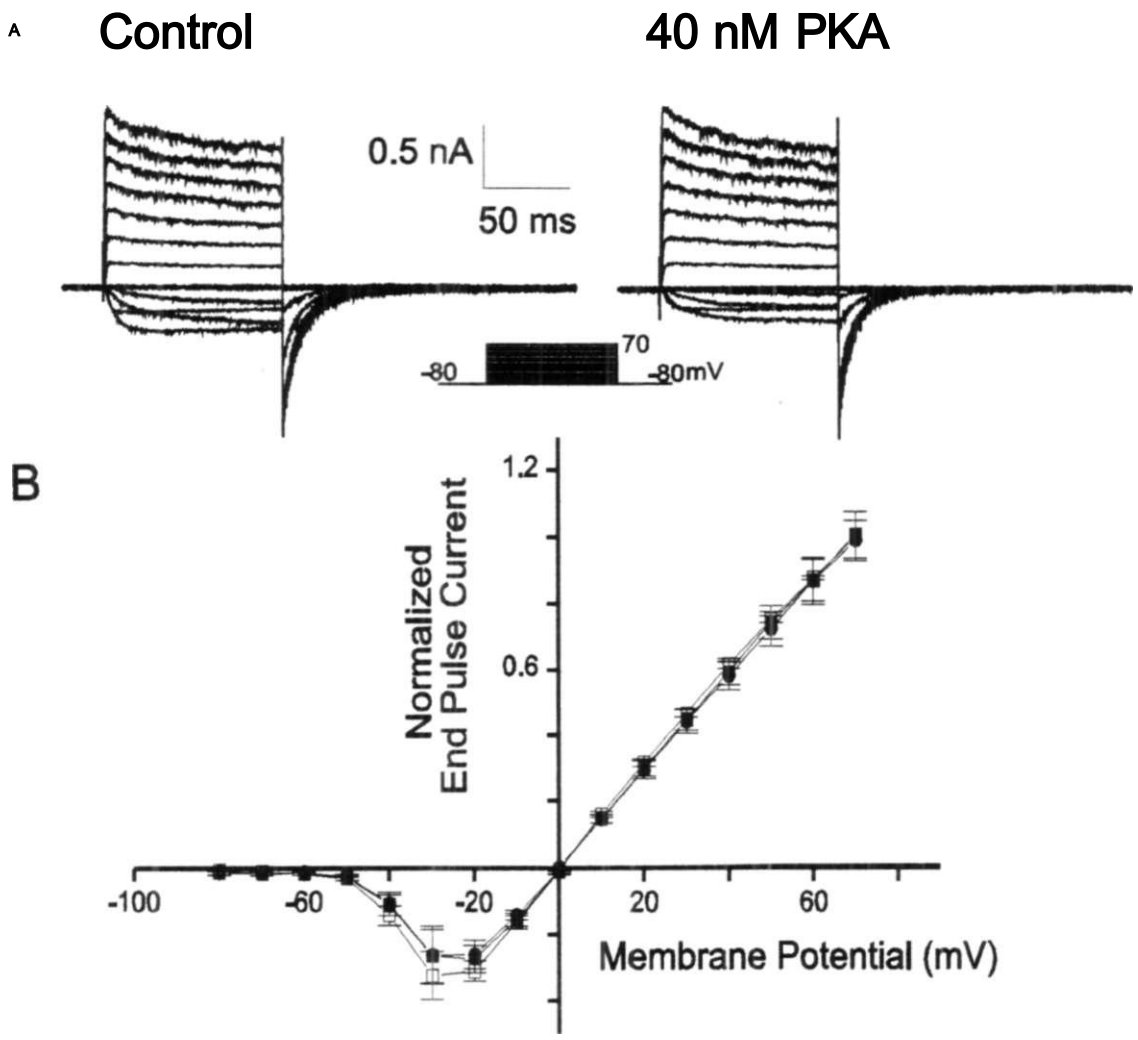


Figure 24: Effect of PKA on Kvl.5 inside-out macropatch currents. A: Representative recording demonstrating the lack of effect of the catalytic subunit of PKA (40 nM) in the presence of ATP (2 mM) on Kvl.5 channels of an inside-out patch (symmetrical 140:140 mM K* recording conditions). **B:** Normalized average I-V relations for 4 patches exposed to 40 nM PKA. *(Open squares - control, closed squares and closed circles - 5 and 10 min PKA application, respectively).*

data obtained in the whole-cell configuration, no effect of csub-PKA was observed on Kv1.5 macropatch current amplitude after 10 min of treatment (Fig. 24).

Secondly, Kv1.5 whole-cell current recordings were performed while dialyzing cells with 74 nM csub-PKA included in the pipette solution. In performing these experiments, the tips of whole-cell patch pipettes were filled with normal (csub-PKA-free) pipette solution, and were then back-filled with pipette solution containing the kinase. These experiments are similar to those performed by Aiello et al. (1995) in studying the regulation of native RPV Kdr currents by PKA, in which a time-dependent decrease in current amplitude was observed during the first 3-5 min of dialysis of the vascular myocytes with the selective peptide inhibitor of PKA, PKI. In cells dialyzed with csub-PKA, an increase in Kv1.5 current amplitude was recorded by depolarizing pulses to +10 mV from a holding potential of -60 mV. An analysis of Kv1.5 whole-cell currents, recorded after 5-10 min of cell dialysis with csub-PKA, demonstrated steady-state activation values with a $\mathbf{V_Q}.5$ = -23.7 ± 0.7 mV and a *k* value of 8.0 ± 0.6 mV (n=5). Both of these values are significantly different from those obtained from Kv1.5 expressing cells in the absence of csub-PKA dialysis (Table 2) ($p<0.05$), and are inconsistent with the lack of effect observed for forskolin and 8Br-cAMP on Kv1.5 whole-cell currents.

In order to determine if Kv1.5 channels could be modulated by dephosphorylation, analogous experiments were performed, in which the protein phosphatase 2A (PP2A) was used to back-fill pipettes. Surprisingly, a similar effect on Kv1.5 current amplitude and steady-state activation was observed with PP2A dialysis as

that observed with csub-PKA dialysis. Kv1.5 expressing cells dialyzed with PP2A for 5-10 min demonstrated a $V_{0.5}$ for steady-state activation of -20.7 ± 0.9 mV and a *k* value of 8.8 ± 0.8 mV (n=4); these values were significantly different from control Kv1.5 whole-cell currents (Table 2) ($p<0.05$), but not from csub-PKA dialyzed cells. An analysis of the buffer content of the purified csub-PKA and PP2A enzyme stocks demonstrated the presence of dithiothreitol (DTT) in both preparations. The enzyme dilutions utilized in these experiments yielded a concentration of 10 pM DTT in the whole-cell pipette solution. To determine if the hyperpolarizing shift in steady-state activation and the change in voltage sensitivity of Kv1.5 channels observed in the csub-PKA and PP2A experiments was due to non-specific effects of the enzyme buffers, csub-PKA buffer solution was diluted into whole-cell pipette solution. Similar to that of csub-PKA and PP2A dialyzed cells, Kv1.5 whole-cell currents dialyzed with buffer alone demonstrated a steady-state activation $V_{0.5}$ value of -20.3 ± 0.4 mV and a *k* value of 7.4 ± 0.3 mV (n=5). These values were determined to be significantly different from control (Table 2), but not from csub-PKA or PP2A dialyzed cells. Therefore, it was concluded that the activation of Kv1.5 channels observed was due to the effects of the reducing agent DTT present in purified enzyme stocks and not to the enzymatic activity of the csub-PKA or PP2A. This is similar to the effects of redox potential on Kv1.4 channel gating reported previously (Stephens et al., 1996).

3.8.2. Kv1.2 homomultimeric channels

Whole-cell currents from HEK cells transiently transfected with Kv1.2 cloned from RPV were recorded and the ability of PKA to modulate Kv1.2 homomultimeric

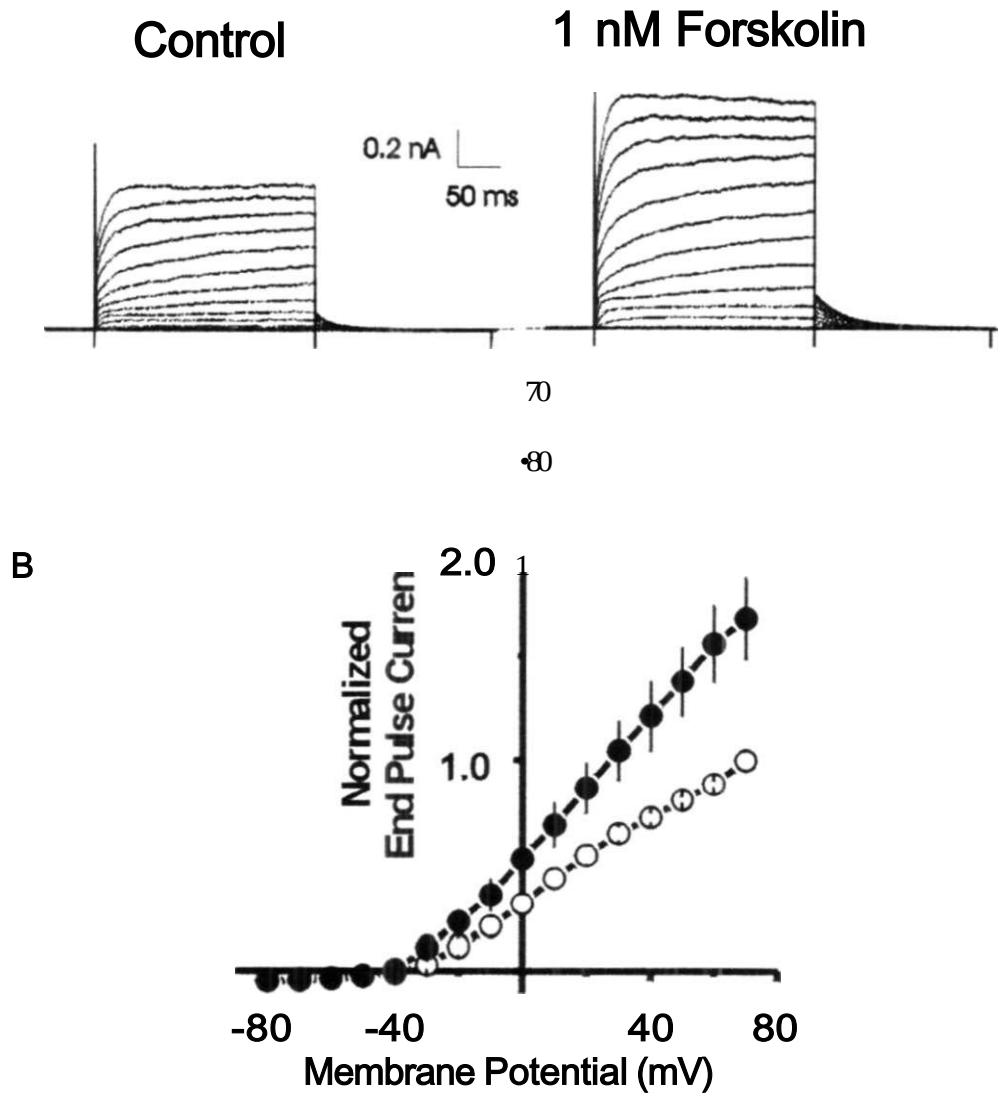


Figure 25: Effect of PKA activation by forskolin on Kv1.2 whole-cell current. A: Representative recordings demonstrating the potentiation of Kv1.2 whole-cell current by 1 uM forskolin. B: Normalized average I-V relation for 6 cells exposed to 1 uM forskolin *(open circles-control, closedcircles-forskolin).* A significant increase in Kv1.2 current at all membrane potentials between -40 and +70 mV was observed ($p<0.05$).

channels was assessed. Application of forskolin (1 uM) resulted in a robust increase in Kvl.2 current amplitude at all voltages between -40 and +70 mV (Fig. 25). This is consistent with the previously reported potentiation of rat Kvl.2 channels expressed in Xenopus oocytes by forskolin and by the application of csub-PKA (Huang et al., 1994). The steady-state activation of Kvl.2 whole-cell currents was unaffected by forskolin with **VQ**.5 values of -4.4 ± 1.5 mV and -5.3 ± 2.2 mV (n=6) for control and after 10 min of forskolin treatment, respectively.

3.9. Summary: Molecular basis of VSM Kv channels

This study demonstrates the expression of Kvl channel proteins Kvl.2, Kvl.4, Kvl.5, Kvp1.2, Kvp1.3, and Kvp2.1 in RPV VSM. Furthermore, it was shown that Kvl.2/Kvl.5 heteromultimeric channel complexes are the predominant 4-AP-sensitive VSM Kdr channels in RPV. This interpretation is based upon complementary molecular biological, immunological, biochemical and pharmacological data. In addition, the interaction of the Kvpi.2 subunit with pore-forming Kvl.5 a subunits in RPV was identified. This is the first demonstration of the association of Kva subunits and modulatory Kvp subunits in smooth muscle. A potentiation of recombinant Kv channels composed of Kvl.2 subunits, but not of Kvl.5 subunits, by activation of PKA is also demonstrated in an attempt to understand the molecular basis of PKA modulation of RPV Kdr current.

CHAPTER 4: MOLECULAR BASIS OF VSM K_{NDP} CHANNELS

4.1. RT-PCR for K^TP channel subunits

The expression pattern of specific K^TP channel subunits in RPV was investigated to determine molecular candidates that may contribute to VSM K^DP channels present in this tissue (Beech et al., 1993b; Cole et al, 2000). Specific primers for the known K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B) were designed based upon alignments of DNA sequences from multiple species present in GenBank. RT-PCR of mRNA isolated from RPV was performed in conjunction with brain mRNA, that was utilized as a positive control confirming the functional properties of all primer pairs and the integrity of each group of RT-PCR reactions performed. RT-PCR demonstrated the expression of mRNA for Kir6.1 (563 bp), Kir6.2 (751 bp), SUR2A (570 bp) and SUR2B (614 bp) in RPV. SUR1 (714 bp) was not detected in RPV even with a second round of PCR (total of 70 cycles), although it was detected in rat brain mRNA utilizing only 35 cycles (Fig. 26). The expression of Kir6.1 and SUR2B mRNA in RPV is consistent with the proposed identity of VSM **KNDP** channels (Yamada et al., 1997), but the role of Kir6.2 and SUR2A in RPV remains to be defined.

4.2. Expression of Kir6.1/SUR2B channels in HEK cells

cDNAs encoding the Kir6.1 and SUR2B subunits were expressed in HEK cells and their regulation by PKC activation was examined. These experiments were performed based upon the following observations: i) mRNA expression of Kir6.1 and

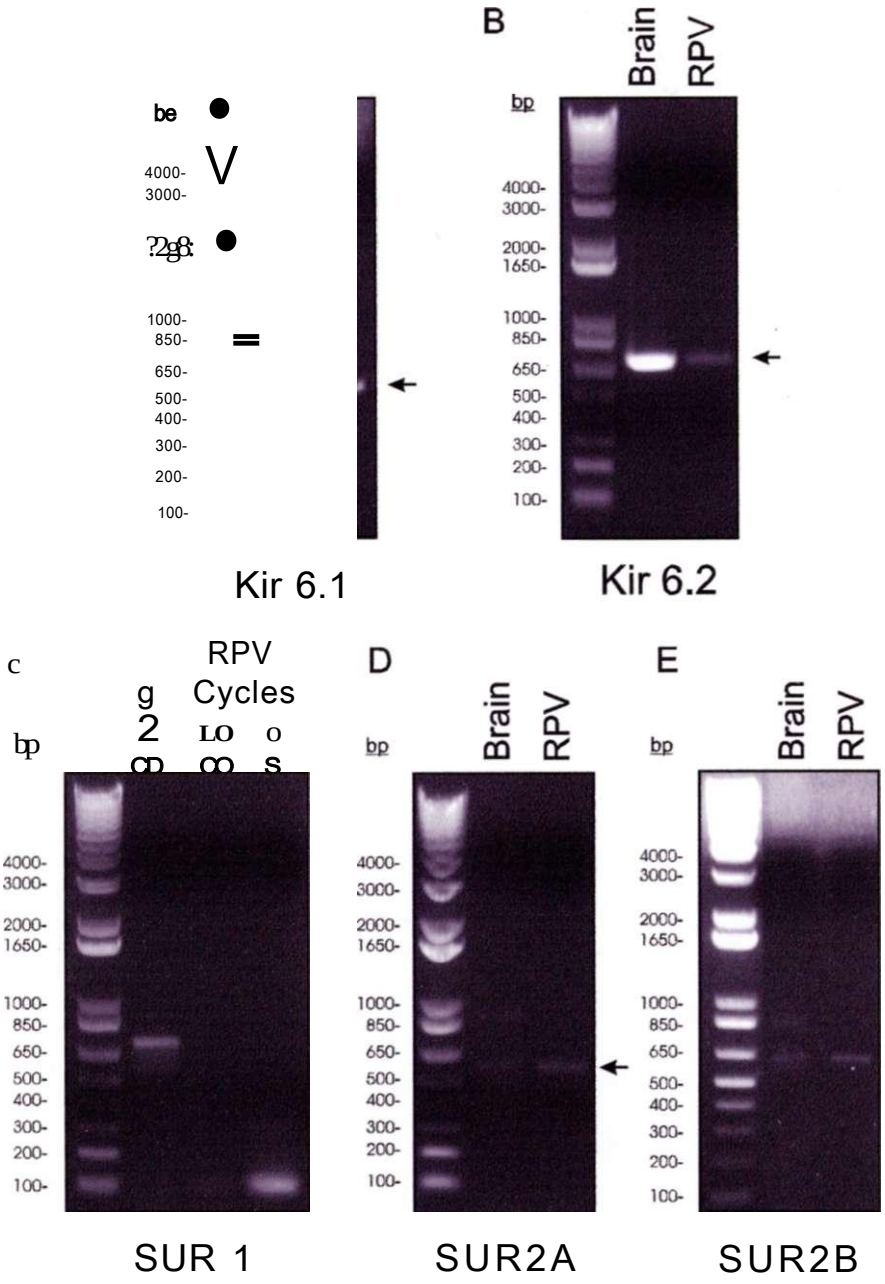


Figure 26: RT-PCR **for KATP subunits in rabbit portal vein** (RPV). A **and** B: RT-PCR for Kir6.1 and Kir6.2. 563 bp and 751 bp products, respectively, detected in rat brain (Brain) and in RPV. C: RT-PCR for SUR1. 714 bp product detected in Brain, but not in RPV after 70 cycles. **D and E:** RT-PCR for SUR2A and SUR2B. 570 bp and 614 bp products, respectively, detected in Brain and in RPV.

SUR2B subunits in PvPV VSM, ii) VSM K_{NDP} channels and Kir6.1/SUR2B channels demonstrate similar biophysical and pharmacological properties (Yamada et al., 1997; Satoh et al., 1998), and iii) the reported modulation of single PvPV VSM K_{ATP} channels by PKC activation (Cole et al., 2000). Kir6.1/SUR2B channels demonstrated robust activation by the K^+ channel opening drug, pinacidil (50 pM), and inhibition by glibenclamide (3 pM) in cell-attached (C-A) membrane patches. NPo values for channel activity demonstrated patch to patch variability due to the inconsistent channel density provided by overexpression of these cloned channel subunits. This is reflected in the average NPo values presented in Table 3. Single channel conductances of 36.5 ± 0.4 pS in C-A patches (n=22) and 36.5 ± 0.8 pS in 1-0 patches (n=13) were recorded at -40 mV, consistent with previous reports for Kir6.1/SUR2B channels (Yamada et al, 1997; Satoh et al., 1998), and similar to the values reported for RPV K_{ATP} channels (41 ± 0.9 pS C-A and 37.3 ± 1.5 pS I-O) (Cole et al, 2000). Figure 27 shows a representative current-voltage relation of single Kir6.1/SUR2B channels recorded from a C-A membrane patch. A small degree of rectification was observed for these channels at more positive potentials, consistent with the established weak inward rectifying properties of Kir6-containing channels and native VSM K_{ATP} channels (Seino, 1999; Cole et al., 2000).

4.3. Effect of PKC activation by phorbol ester on Kir6.1/SUR2B channels

In C-A patches, Kir6.1/SUR2B channels were activated by 50 pM pinacidil and demonstrated a time-dependent decline in open probability (NPo) during subsequent treatment with the PKC activator, PdBu (50 nM) (Fig. 28). Average NPo values for

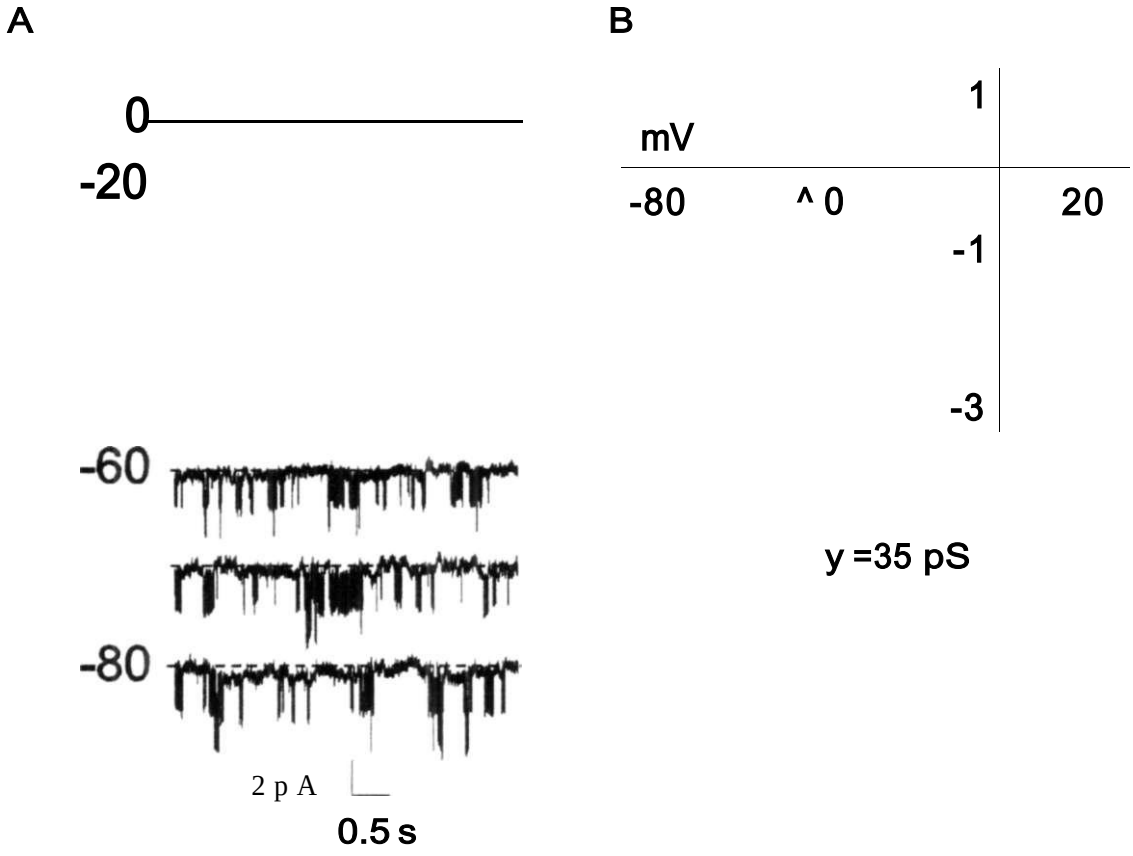


Figure 27: Kir6.1/SUR2B channel i-V relationship. A: Representative single-channel recording of a C-A patch from a HEK cell expressing Kir6.1/SUR2B channels with 140 mM K* in the pipette. **B:** Representative linear i-V plot of channels in A, demonstrating a single-channel conductance of 35 pS.

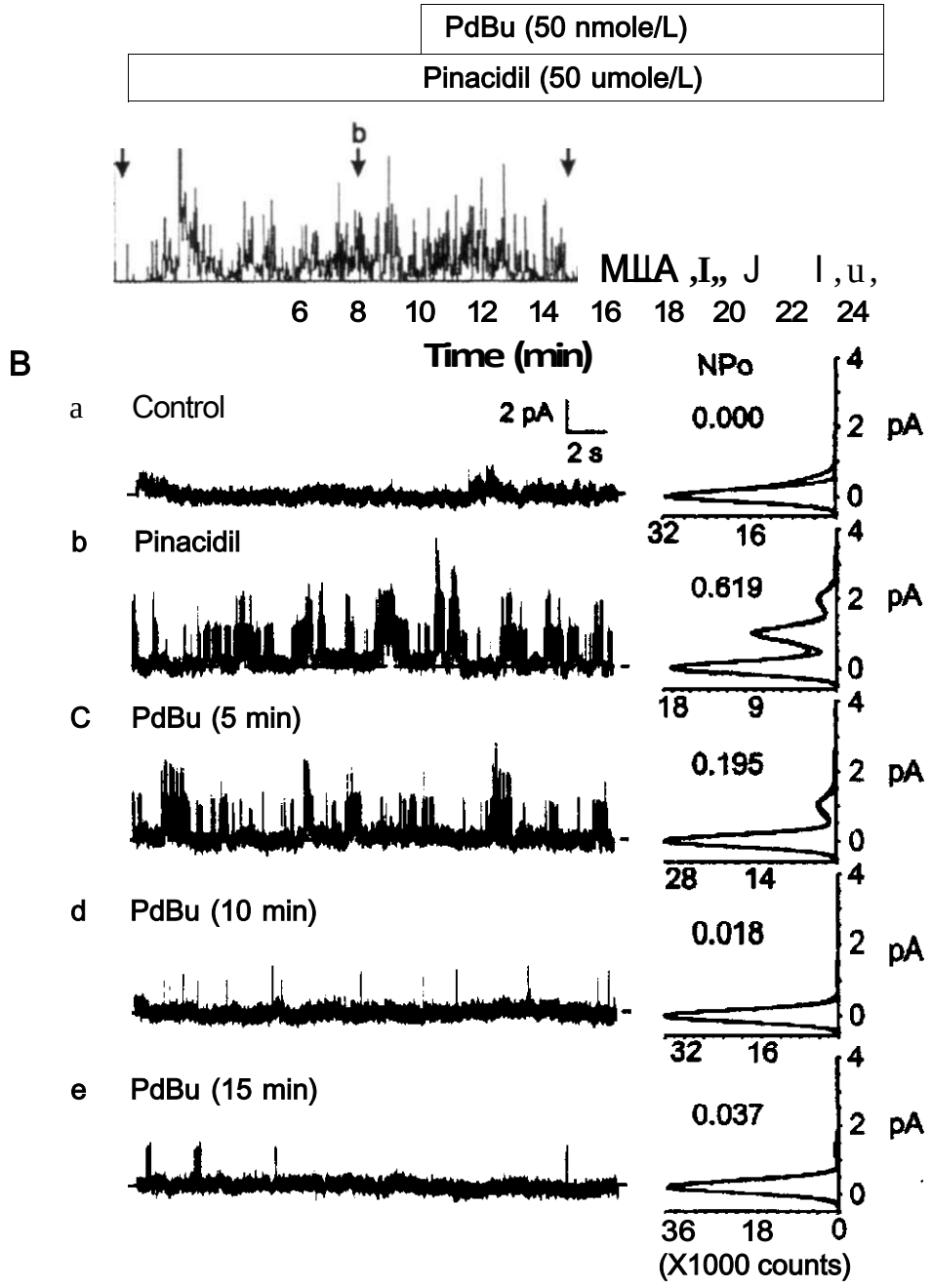


Figure 28: Effect of PdBu on Kir6.1/SUR2B channel activity. A: NPo versus time for Kir6.1/SUR2B channels in a C-A patch demonstrating activation with 50 pM pinacidil and a time-dependent decline in activity following application of 50 nM PdBu. **B:** Representative 15 s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-e in panel A.

Table 3: Effect of PKC activation on NPo of recombinant Kir6.1/SUR2B channels.

Experiment	n	Treatment	N£o
1. Pinacidil/PdBu	6	Control	0.026 ± 0.022
		Pinacidil (50 p:mole/L)	0.616 ±0.240
		PdBu (50 nmole/L) + Pinacidil	**0.017 ± 0.030 ***
2. Pinacidil/PdDe		Control	0.000 ± 0.000
		Pinacidil (50 |lmole/L)	0.191 ±0.089
		PdDe (50 nmole/L) + Pinacidil	0.878 ± 0.392
3. Pinacidil/PdBu		Control	0.000 ± 0.000
chelerythrine		Pinacidil (50 (imole/L)	0.083 ± 0.027
pretreatment		PdBu (50 nmole/L) + Pinacidil	0.141 ±0.066
4. Pinacidil/Ang II	5	Control	0.000 ± 0.000
		Pinacidil (50 (imole/L)	0.455 ±0.175
		Ang **II** (0.1 (Imole/L) + Pinacidil	**0.114 ±0.099***
5. Pinacidil/Ang II	6	Control	0.000 ± 0.000
chelerythrine		Pinacidil (50 ^imole/L)	0.666 ± 0.348
pretreatment		Ang **II** (0.1 [imole/L) + Pinacidil	0.780 ± 0.466
6. PKC		Control	0.766 ± 0.277
		PKC (7.5 nmole/L)	**0.017 ± 0.003***
		Washout	0.275 ±0.120
7. PKC/pep(19-31)	4	Control	0.275 ± 0.091
		PKC (7.5 nmole/L)+pep(19-31) (5 |imole/L)	0.226 ±0.146
		PKC (7.5 nmole/L)	**0.037 ±0.031***
		Washout	0.199 ±0.101*

* indicates significantly different from value in pinacidil (experiments 1 - 5) or control (experiments 6 - 7).

pinacidil and following 15 min of pinacidil plus PdBu treatment were 0.616 ± 0.240 and 0.017 ± 0.030, respectively (n=6) (Table 3). In contrast, application of the inactive phorbol ester, PdDe, had no effect on pinacidil-induced Kir6.1/SUR2B channel activity (Fig. 29). Average NPo values for pinacidil and after 15 min of treatment with pinacidil plus PdDe were 0.191 ± 0.089 and 0.878 ± 0.392, respectively (n=5) (Table 3). Pre-treatment for 10-15 min with the PKC selective inhibitor, chelerythrine (3 pM), after obtaining the C-A configuration, did not affect pinacidil-induced Kir6.1/SUR2B channel activity, but suppressed the reduction in NPo following PdBu treatment (Fig. 30). Average NPo values for pinacidil and after 15 min of treatment with pinacidil plus PdBu in the presence of chelerythrine were 0.083 ± 0.027 and 0.141 ± 0.066, respectively (n=5) (Table 3).

4.4. Effect of angiotensin II activation of PKC on Kir6.1/SUR2B channels

Previously, RPV VSM Kfsjrjp channel activity demonstrated inhibition by angiotensin II via the activation of PKC (Cole et al, 2000). In this study, a rabbit ATj receptor clone was co-expressed along with Kir6.1 and SUR2B subunits in HEK cells, as the AT] receptor subtype is well-known to couple to PKC in RPV, as well as in other cell types (Clement-Chomienne et al, 1996; Muscella, 2000). HEK cells co-transfected with the AT] receptor, Kir6.1 and SUR2B were treated with angiotensin II (100 nM) subsequent to channel activation with pinacidil. Treatment with angiotensin II in the continued presence of pinacidil resulted in a decreased NPo in C-A patches that was significant at 15 min of treatment, but not at 5 min of treatment (Fig. 31, $p<0.05$).

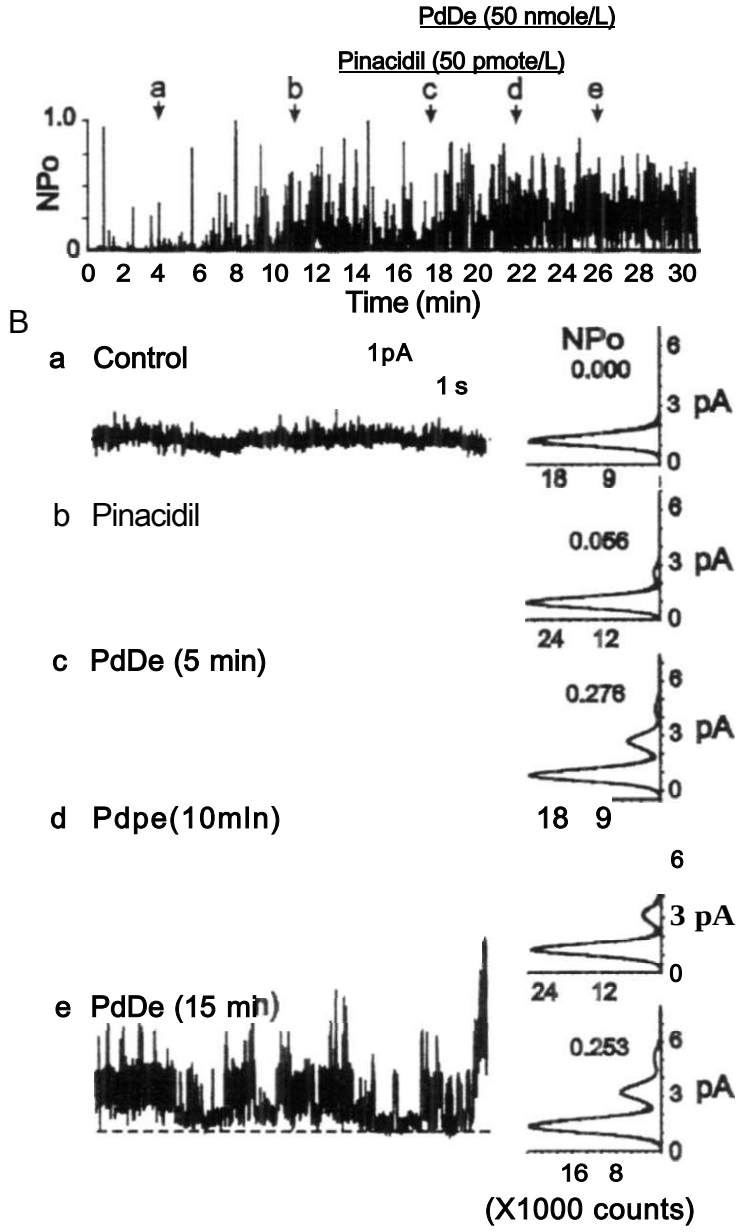


Figure 29: Effect of PdDe on Kir6.1/SUR2B channel activity. A: NPo versus time for Kir6.1/SUR2B channels in a C-A patch, demonstrating activation with 50 uM pinacidil and no effect of 50 nM PdDe over 20 min of application. **B:** Representative 10 s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-e in panel A.

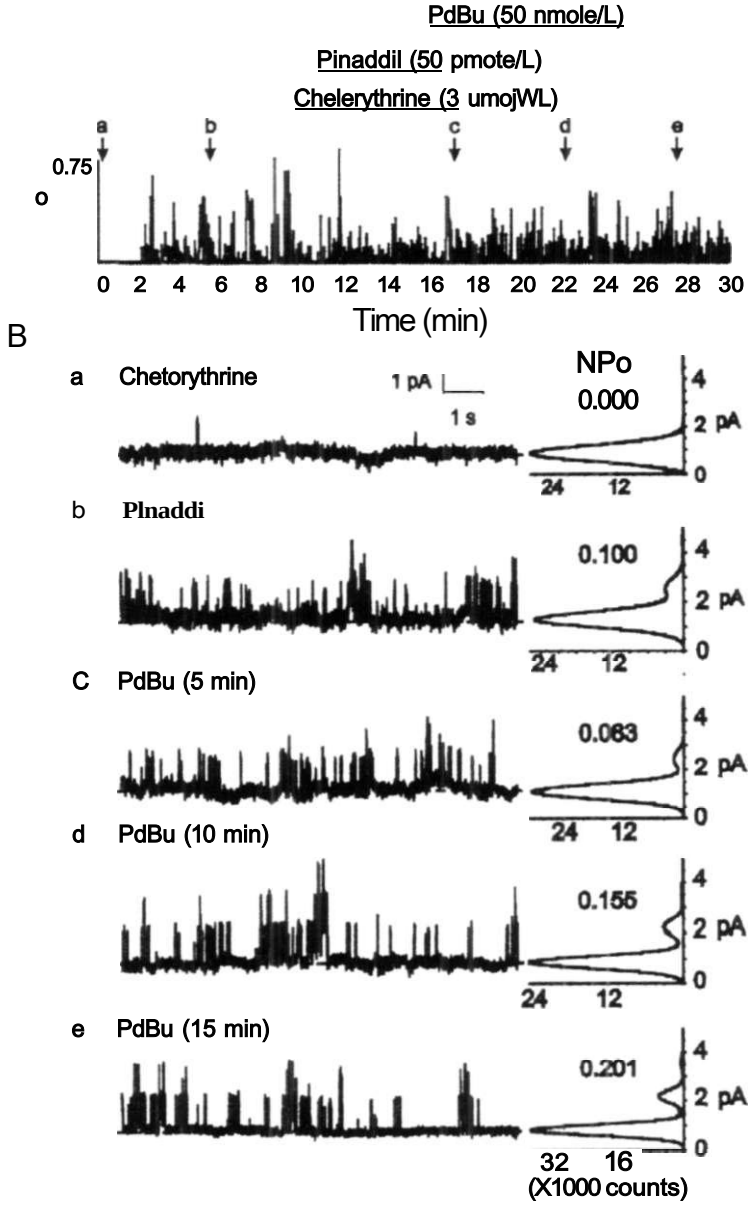


Figure 30: Effect of PdBu on Kir6.1/SUR2B channel activity following chelerythrine pre-treatment. A: NPo versus time for Kir6.1/SUR2B channels in a C-A patch pre-treated with 3 pM chelerythrine, demonstrating activation with 50 pM pinacidil and no effect of 50 nM PdBu over 18 min of application. **B:** Representative 10 s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-e in panel A.

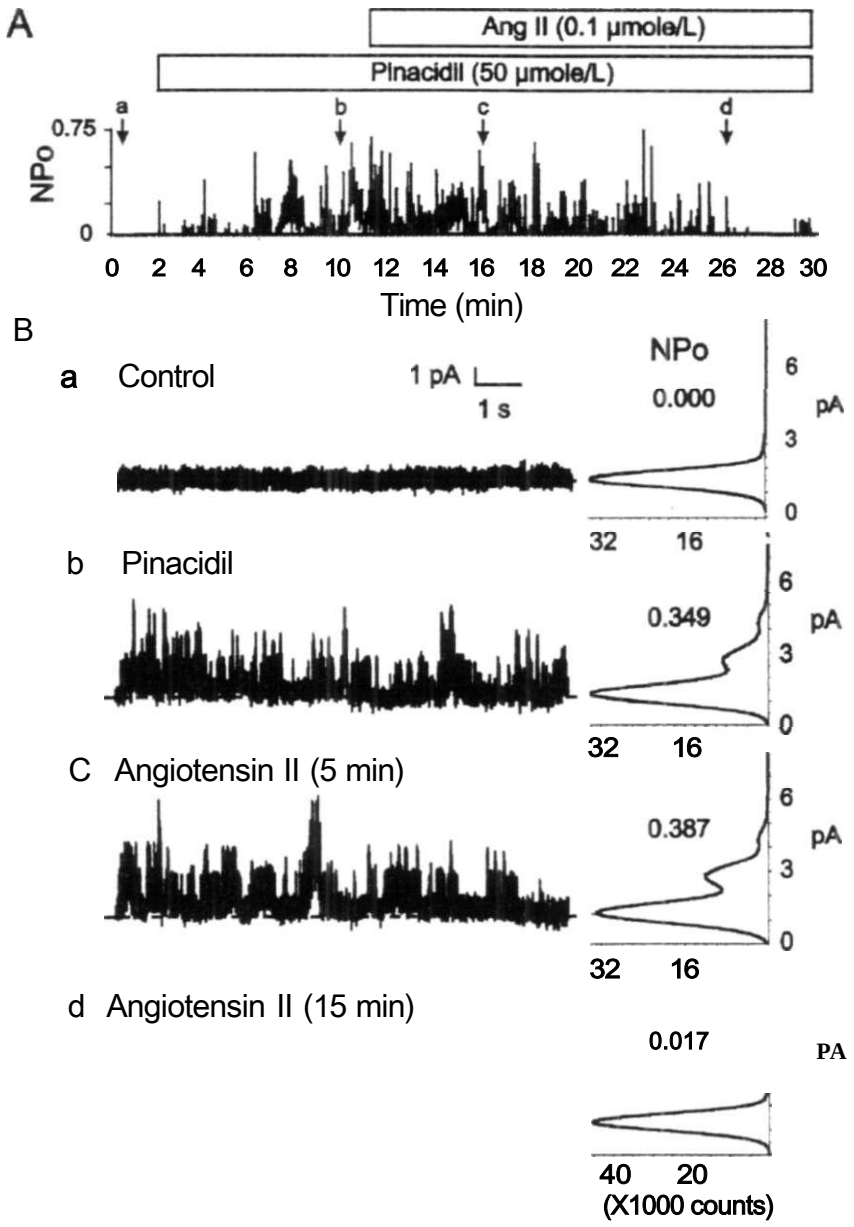


Figure 31: Effect of angiotensin II on Kir6.1/SUR2B channel activity. A: NPo versus time for Kir6.1/SUR2B channels in a C-A patch of a cell co-transfected with the AT, receptor and Kir6.1, SUR2B channel subunits. Channel activation demonstrated following 50 pM pinacidil and a decline in channel activity after 15 min of 0.1 pM angiotensin **II** application. B: Representative 10 s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-d in panel A.

Average NPo values of pinacidil and 15 min treatment with angiotensin II in the continued presence of pinacidil were 0.455 ±0.175 and 0.114 ± 0.099, respectively (n=5) (Table 3). Pre-treatment of HEK cells with chelerythrine, as described for the PdBu experiments, prevented the angiotensin II-induced decrease in Kir6.1/SUR2B NPo (Fig. 32). Average NPo values in pinacidil and after 15 min treatment with angiotensin II plus pinacidil following chelerythrine pre-treatment were 0.666 ± 0.348 and 0.780 ± 0.466, respectively (n=6) (Table 3).

4.5. Effect of PKC on Kir6.1/SUR2B channels in inside-out patches

Kir6.1/SUR2B channel currents were recorded in 1-0 membrane patches of HEK cells in the presence of 0.5 mM ATP and 0.5 mM **ADP** in the bathing solution, conditions which resulted in the physiological activation of channels without the use of pinacidil. This is consistent with the properties reported previously for Kir6.1/SUR2B channels (Yamada et al., 1997; Satoh et al., 1998) and VSM **KNDP** channels (Zhang and Bolton 1995, 1996; Cole et al, 2000). In a first set of experiments, the ability of purified PKC to inhibit Kir6.1/SUR2B channels was tested by applying PKC (7.5 nM) to the cytoplasmic face of I-O patches in the continued presence of **ADP** and ATP. This resulted in a decrease in Kir6.1/SUR2B NPo that was reversed upon washout of PKC with ADP- and ATP-containing solution. Average NPo values for this set of experiments were: control 0.766 ± 0.277, PKC 0.017 ± 0.003, and washout 0.275 ±0.120 (n=4) (Table 3).

In a second set of experiments PKC was applied to 1-0 patches of Kir6.1/SUR2B channels in the presence of the pseudosubstrate inhibitor peptide PKC(19-31), to confirm

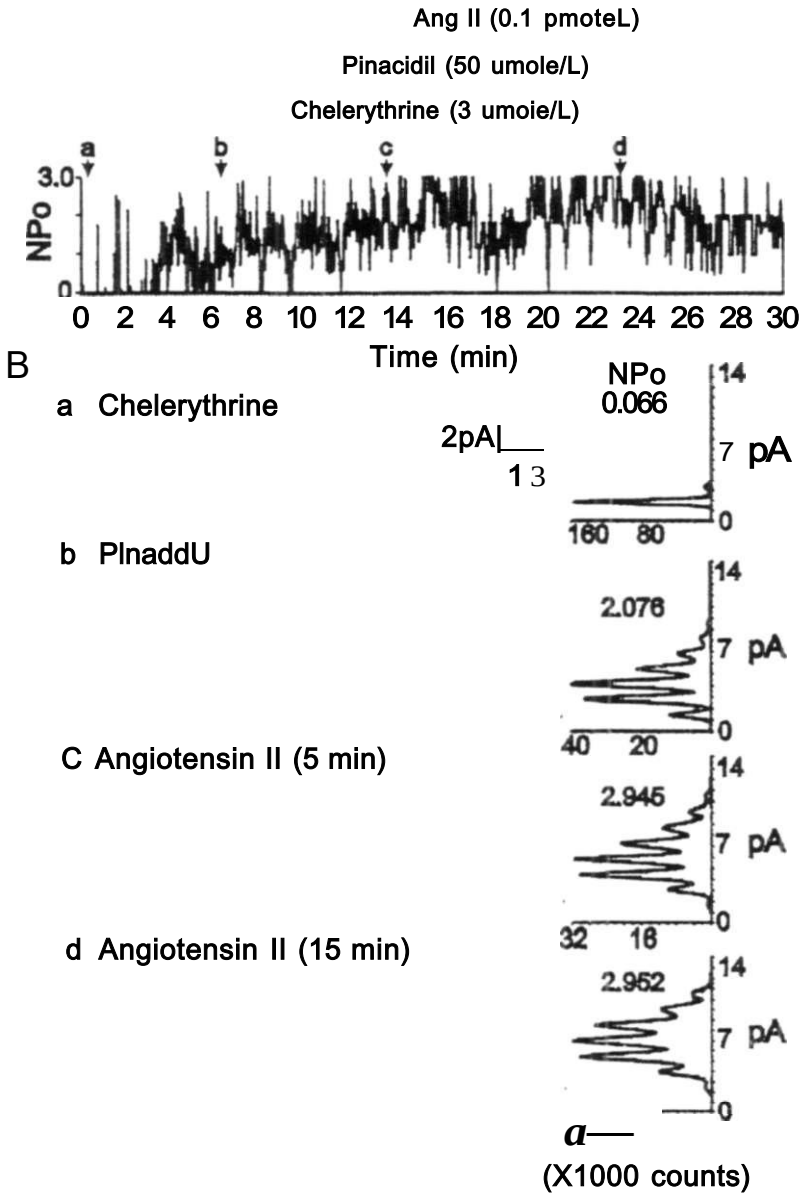


Figure 32: Effect of angiotensin II on Kir6.1/SUR2B channel activity following chelerythrine pre-treatment. A: NPo versus time for Kir6.1/SUR2B channels in a C-A patch co-transfected with the AT, receptor. Channel activation demonstrated following 50 pM pinacidil and no effect of 0.1 uM angiotensin II over 20 min of application. **B:** Representative 10s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-d in panel A.

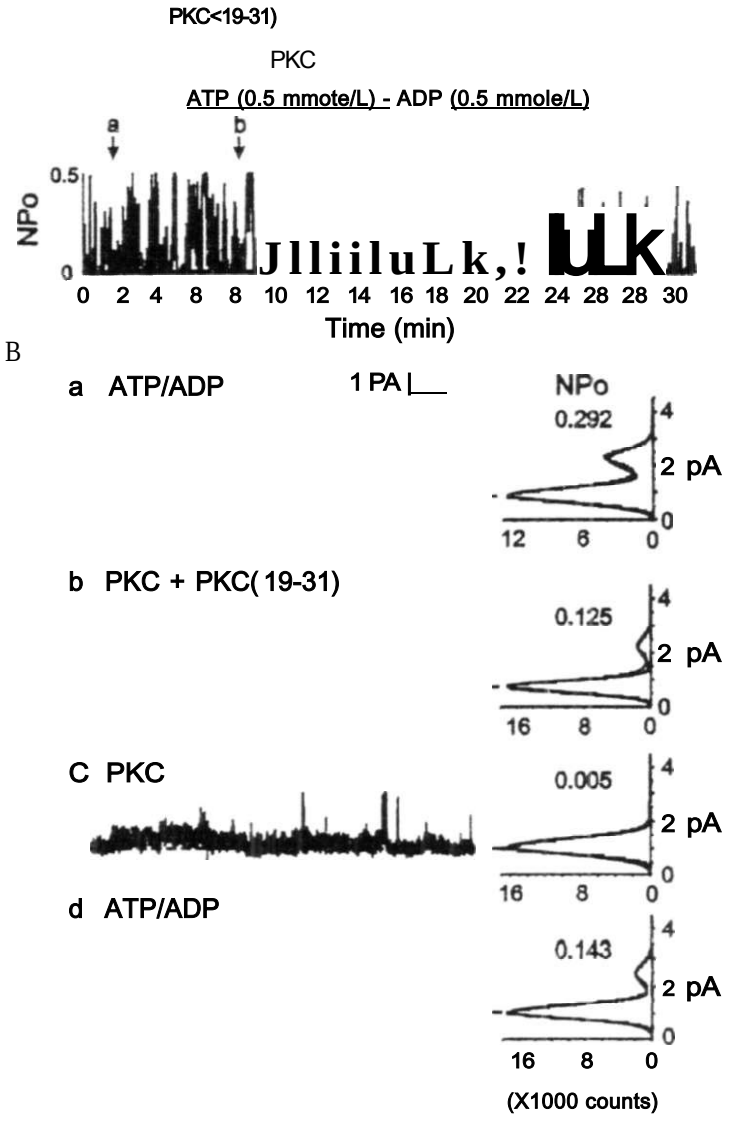


Figure 33: Effect of PKC on Kir6.1/SUR2B channel activity. A: NPo versus time for Kir6.1/SUR2B channels in an 1-0 patch. Channels demonstrated activation by 0.5 mM ATP and 0.5 rnM ADP, no effect of 7.5 nM PKC in the presence of 5 pM pseudosubstrate peptide inhibitor of PKC. PKC(19-31), and subsequent inhibition of activity by PKC applied alone that was reversed upon return to control solution containing 0.5 mM ATP and 0.5 mM ADP. B: Representative 10 s traces, amplitude histograms (based upon 1 min intervals) and corresponding NPo values taken at points a-d in panel A.

that the inhibition of Kir6.1/SUR2B channel activity by PKC was due to the enzymatic activity of the kinase. Kir6.1/SUR2B channels were activated by patch excision into 0.5 mM ADP and 0.5 mM ATP bathing solution. Purified PKC was then applied in the continued presence of ADP and ATP with the inclusion of 5 uM PKC(19-31). No effect on Kir6.1/SUR2B channel activity was observed with PKC plus PKC(19-31) peptide, but subsequent channel inhibition was demonstrated during PKC treatment without PKC(19-31) (Fig. 33). Kir6.1/SUR2B channel activity also demonstrated a reactivation on washout of PKC (Fig. 33), consistent with the first set of experiments and suggestive of the association of a phosphatase with these 1-0 membrane patches. Average NPo values for the second set of 1-0 experiments were: control 0.275 ± 0.091, PKC plus peptide 0.226 ± 0.146, PKC alone 0.037 ± 0.031, and washout 0.199 ± 0.101 (n=4) (Table 3). These experiments show a direct inhibition of Kir6.1/SUR2B channels by the catalytic action of PKC under conditions of channel activation by ADP and ATP.

4.6. P K C **inhibition of Kir6.1/SUR2B by increasing inter-burst interval**

PKC inhibition of native V S M R P V K ^ Q p channels was demonstrated to occur via a mechanism increasing inter-burst interval of the K ^ ^ p channels (Cole et al., 2001). An analysis of the burst kinetics of Kir6.1/SUR2B channels in this study, with and without PdBu treatment in the continued presence of pinacidil, also implicates an increase in inter-burst interval in the mechanism of PKC modulation of Kir6.1/SUR2B channels (Table 4). Burst duration was unaffected by PdBu treatment. Average burst duration values for pinacidil and pinacidil plus PdBu of 57.8 ± 4.4 ms and 57.9 ± 4.6 ms.

Table 4: Effect of PKC activation on recombinant Kir6.1/SUR2B channel burst kinetics.

Treatment	Burst Duration (ms)*		Intra-burst dwell times (ms)**	
	n		n	Open	Closed
l.Pinacidil	155	57.8±4.4	37972	1.71 ±0.27	0.46±0.12
2. **PdBu/Pinacidil**	157	57.9 ±4.6	42023	1.81±0.24	0.44 ±0.14

* analysis based on burst duration for 155 and 157 individual bursts (n) in three patches in the absence (pinacidil alone) and presence of PdBu + pinacidil, respectively.
** analysis based on 37972 and 42023 transitions in >1000 bursts in four patches in the absence (pinacidil alone) and presence of PdBu + pinacidil respectively.

respectively, were determined based on an analysis of > 150 bursts from 3 patches in each treatment group. Intra-burst open and closed dwell times were also unaffected by PdBu based on an analysis of > 1000 bursts from 4 different patches in each condition. Average open and closed dwell times were 1.71 ± 0.27 ms and 0.46 ± 0.12 ms for pinacidil, and 1.81 ± 0.24 ms and 0.44 ± 0.14 ms for pinacidil plus PdBu, respectively (Table 4). The high expression level of the Kir6.1/SUR2B channels in transfected HEK cells made it impossible to measure inter-burst interval directly, due to the presence of multiple channels in all patches obtained. Nonetheless, the lack of change in burst duration and intra-burst kinetics indicates an increase in the inter-burst interval as the most likely explanation for the mechanism of PKC-mediated decrease in NPo of Kir6.1/SUR2B channels in this study.

4.7 Summary: molecular basis of VSMKjynp channels.

The first comprehensive expression pattern analysis of mRNAs encoding K^jp channel subunits in VSM was performed in this study. These results demonstrate the expression of Kir6.1, Kir6.2, SUR2A and SUR2B subunits in RPV VSM. This study also demonstrates an inhibition of K^jp channels composed of Kir6.1 and SUR2B subunits by activation of AT} receptors and PKC. The results indirectly demonstrate a lengthening of inter-burst interval of Kir6.1/SUR2B channel activity as the mechanism involved in PKC modulation of channel gating. K^TP channels composed of Kir6.1/SUR2B subunits are, therefore, sufficient to reconstitute the reported inhibitory

effects of PKC on native V S M K ^ p channel activity that occur via a lengthening the inter-burst interval.

CHAPTER 5: DISCUSSION

In this study, the molecular bases of **K ^ Q P** and 4-**AP**-sensitive Kv channels of **R P V V S M** myocytes were investigated. **A** combination of molecular biological, biochemical and electrophysiological techniques was utilized to determine the expression pattern of genes encoding channel subunits and to provide functional data implicating a role for specific subunits in the formation of native channel complexes.

5.1. VSM Kv channels.

5.1.1. Expression and assembly pattern of RPV Kv channels.

This study provides the first evidence for the co-assembly of Kv channel a and P subunit proteins into heteromultimeric channel complexes composed of Kvl.2, Kvl.5, and Kvp 1.2 subunits in **V S M .** In addition, pharmacological tools in conjunction with co-**IP** experiments indicate that the majority of the 4-**AP**-sensitive Kdr current of **R P V** myocytes is due to Kvl.2/Kvl.5 heteromultimeric channel activity. The production of novel antibodies also enabled the first detection of specific splice variant proteins of the Kvpi gene in isolated **V S M** cells.

The biophysical properties and 4-**AP** sensitivity demonstrated by **R P V** Kv current (Beech and Bolton, 1989a; Clement-Chomienne et al., 1999; Kerr et al., 2001) are consistent with the properties of Kvl channels, but not Kv2-Kv4 channels of the Kv channel superfamily. On the basis of functional similarity, it was postulated that native. 4-AP-sensitive components of Kv current in **R P V** myocytes were likely due to the

expression of underlying Kvl channel subunits. For this reason, the expression and assembly pattern of Kvla and Kv(3 subunits was examined. RT-PCR demonstrated mRNA expression of Kvl.2, Kvl.4 and Kvl.5, as well as the Kvp subunits Kvp1.1, Kvp1.2, Kvp1.3, Kvp2.1 and Kvp2.2, but did not detect the expression of Kvl.l, Kvl.3, Kvl.6, KvP3.1 or Kvp4 subunits (Figs. 5 and 6). Commercial Kvla subunit antibodies, and rabbit polyclonal antibodies raised against Kvp subunits detected in RPV at the mRNA level, were initially tested in transfected HEK cells for their specific immunoreactivity and functional ability in Western blotting, immunocytochemical and immunoprecipitation experiments (Figs 9, 10, 13-17, 19 and 20). The demonstrated ability of an antibody to perform these experiments in HEK cells was required prior to performing experiments on RPV proteins, thereby ensuring the specificity of antibodies in RPV experiments. KvP subunit family members, Kvp1.2, KvP1.3 and KvP2.1 proteins, were detected, along with Kvl.2, Kvl.4 and Kvl.5 a subunit proteins in RPV (Figs. 12, 18 and 21), of which the expression of Kvl.2, Kvl.4, Kvl.5, Kvpl.2 and Kvp1.3 proteins was identified within freshly isolated RPV vascular myocytes (Fig 18). These results are significant in that previous immunocytochemical studies of Kv subunit expression in isolated VSM cells have utilized cultured smooth muscle cells (Xu et al.. 1999, 2000), in which Kv channel expression is altered (Tang and Wang, 2001).

Co-immunoprecipitation experiments were conducted, directly demonstrating the association of Kvl.2 and Kvl.5 subunits within heteromultimeric Kv channels of RPV. The association of Kvl.5 and Kvp1.2 subunits was also detected via their reciprocal co-immunoprecipitation. demonstrating the first association of modulatory Kvp subunits

with pore-forming a subunits in VSM and providing the first direct evidence for heteromultimeric channel assembly in smooth muscle. The predominance of the heteromultimeric association of Kv1.2 and Kv1.5 subunits in RPV was then demonstrated through the use of the Kv channel blockers 4-AP and charybdotoxin, altering native RPV Kdr current and/or HEK cells expressing channels composed of Kv1.2 and Kv1.5 subunits.

5.1.2. **Expression of** Kv **channel subunits in other smooth muscles**

The expression of Kv1.2, Kv1.4 and Kv1.5 a subunit proteins in RPV is consistent with the widespread expression of these three subunits in vascular (Archer et al., 1998; Yuan et al., 1998; Clement-Chomienne et al., 1999; Hulme et al, 1999; Xu et al., 1999, 2000; Ohya et al., 2000; Cox et al, 2001; Lu et al., 2001), airway (Adda et al., 1996; Wade et al., 1999) and gastrointestinal smooth muscles (Epperson et al., 1999). However, mRNAs encoding Kv1.1, Kv1.3 and Kv1.6 were not detected in RPV, but then-expression in other smooth muscles has been reported (Adda et al., 1996; Archer et al.. 1998; Yuan et al., 1998; Epperson et al., 1999; Xu et al., 1999; Cox et al., 2001). This tissue-specific expression of Kv1.1, Kv1.3 and Kv1.6 may represent specific differences in the expression pattern of ***a*** subunits, providing subtly different Kv current properties to match different functional requirements in these tissues. Alternatively, the detection of Kv1.1, Kv1.3 and Kv1.6 subunits may be due to their expression in contaminating cell types, and not from the smooth muscle myocytes in the preparations. For example, the detection of Kv1.1 mRNA in airway (Adda et al.. 1996), rat aorta, mesenteric, pulmonary and tail artery (Yuan et al.. 1998; Cox et al., 2001) smooth muscle tissues may represent

the detection of this a subunit in contaminating neurons within these smooth muscle tissues (Rhodes et al, 1997; Shamotienko et al., 1997; Coleman et al., 1999), or in other specialized cell types, such as interstitial cells of Cajal. that express Kv1.1 and are present in gastrointestinal smooth muscle tissues (Epperson et al., 1999; Hatton et al., 2001). Kv1.3 is highly expressed in lymphocytes (Lewis and Cahalan, 1995) and has been detected at the mRNA level in aorta, mesenteric and tail artery smooth muscle (Xu et al., 1999; Cox et al, 2001). The detection of Kv1.3 in these preparations could be the result of lymphocyte infiltration into the smooth muscle layers of the blood vessel wall. The possibility that the detection of Kv1.1 and Kv1.3 is due to contaminating cell types within smooth muscle tissues is consistent with the low levels of Kv1.1 and Kv1.3 mRNAs detected in aorta, mesenteric and tail arteries compared to the significantly higher levels of mRNA encoding Kv1.2 and Kv1.5 (Cox et al., 2001). Kv1.6 is expressed in all parts of the gastrointestinal tract (Epperson et al., 1999) and has been detected at the mRNA (Yuan et al., 1998) and protein levels in pulmonary artery (Archer et al., 1998), but was not detected here in RPV smooth muscle. The basis for this apparent tissue-specific expression is unknown, and in the absence of any confirmation of protein expression in isolated myocytes, or a functional role for the Kv1.6 subunit. it is not possible to reach any firm conclusions regarding the presence of the subunit in VSM.

Consistent with the expression of Kvp subunit mRNA in rat aorta, pulmonary, mesenteric and tail artery smooth muscle (Yuan et al., 1998, Xu et al, 1999; Cox et al.. 2001), mRNA encoding the three splice variants of KvP1, and two splice variants of Kvp2 demonstrated expression in RPV. Specific splice variants of Kvpi, Kvp1.1 and

Kvp 1.2, were found in smooth muscle cells of certain regions of the gastrointestinal tract, but KvP2.1 expression was not detected (Epperson et al., 1999), consistent with the lack of Kvp2.1 expression observed in **VSM** tissues by Cox et al. (2001). The comprehensive expression analysis of all known Kvp subunits provided by this study, including the specific splice variants of the Kvpi and KvP2 genes, is a first in smooth muscle. In addition, the demonstrated protein expression of Kvpi.2, Kvp 1.3 and Kvp2.1 subunits in PvPV is the first identification of Kvp protein expression in smooth muscle.

In a number of studies analyzing the expression of Kv subunits, protein detection was not demonstrated to support Kv subunit mRNA expression that was shown in intact tissue samples. As noted above, tissue samples are frequently a source of not only smooth muscle cell mRNA, but also of mRNA from other cell types. In addition, several studies have employed cultured vascular smooth muscle cells for RT-PCR (Yuan et al., 1998) or protein detection by immunocytochemistry (Xu et al., 1999, 2000). This approach is inappropriate, due to demonstrated changes in Kv currents upon cell culturing of vascular myocytes (Tang and Wang, 2001). As well, few previous studies of VSM have made a convincing attempt to correlate the expression of individual Kv subunit proteins with defined biophysical, pharmacological and/or functional properties of native VSM currents.

5.1.3. Pharmacological and biophysical evidence for RPV Kv channels composed of Kvl.4, Kvl.2 and Kvl.5 subunits.

The biophysical and pharmacological properties of expressed subunits can provide information with respect to the composite molecular identity of native Kv

channels. For example, channels composed of Kv1a family members were implicated as contributors to the 4-AP-sensitive Kv current in RPV myocytes, due to their similar biophysical properties and 4-AP sensitivity to native RPV Kv currents, properties not shared by Kv2-Kv4 channels.

5.1.3.1. Kv1.4 and RPV Kto current.

The expression of the fast inactivating Kv1.4 channel subunit in RPV myocytes is consistent with the biophysical properties of the Kto current previously identified in RPV myocytes (Beech and Bolton, 1989a; Po et al., 1993; Lee et al., 1996; Judge et al.. 1999). The RPV Kto current resembles the A-current of neurons, for which there is strong evidence that Kv1.4-containing channel complexes underlie the current (Sheng et al., 1993). RPV Kto current is more apparent in whole-cell current recordings when a more negative holding potential is employed (i.e. -80 mV vs -60 mV), due to steady-state inactivation of the channels observed at potentials positive to -80 mV (Beech and Bolton, 1989a). RPV Kto also demonstrates an activation threshold close to -65 mV (Beech and Bolton, 1989a). These properties are almost identical to the reported steady-state inactivation and activation properties of Kv1.4 (Po et al., 1993; Lee et al., 1996; Judge et al., 1999), but inconsistent with the properties of other fast inactivating Kv3 and Kv4 channels, which demonstrate activation thresholds at considerably more positive membrane potentials, Em > -10 mV and -40 mV, respectively (Fiset et al., 1997; Franqueza et al., 1999; Weiser et al, 1994). The predominant Kv current in RPV vascular myocytes is a slowly inactivating Kdr type current (Beech and Bolton, 1989b; Aiello et al, 1995, 1998; Clement-Chomienne et al., 1999; Ken- et al, 2001). Therefore, based

upon the ability of one Kv1.4 subunit in a channel complex to evoke fast inactivation (Po et al, 1993; Lee et al., 1996), it is likely that Kv1.4 subunits contribute to only a minority of the Kv channel complexes in RPV, those responsible for the Kto current and not the Kdr current.

5.1.3.2. Kv1.2, Kv1.5 and RPV Kdr current.

The expression of Kv1.2 and Kv1.5 in RPV myocytes, along with the lack of expression of other delayed rectifier type Kv1 subunits, Kv1.1, Kv1.3 and Kv1.6, suggests that Kv1.2 and Kv1.5 pore-forming subunits are the dominant subunits responsible for the 4-AP-sensitive RPV Kdr current. The association of Kv1.2 and Kv1.5 subunits via their co-immunoprecipitation (Fig. 21) indicates the potential contribution of RPV channel complexes composed of homomultimers of Kv1.2, homomultimers of Kv1.5 and/or heteromultimers of Kv1.2 and Kv1.5, to 4-AP-sensitive Kdr channels of RPV myocytes.

Pharmacological evidence, utilizing 4-AP and charybdotoxin (a selective blocker of Kv1.2 homomultimers (Russell et al., 1994)) to inhibit cloned Kv1.2, Kv1.5 and native RPV Kdr whole-cell currents, provides strong evidence for the majority of RPV Kdr channels being composed of heteromultimers of Kv1.2 and Kv1.5 subunits (Fig. 22) (Kerr et al., 2001). RPV Kdr demonstrated a lack of charybdotoxin sensitivity, thereby showing a lack of Kv1.2 homomultimeric channels contributing to the current (Kerr et al., 2001). Block of RPV Kdr by 4-AP is coincident with a positive shift in the steady-state activation of the Kdr current (Kerr et al., 2001). A similar shift is not observed with homomultimers of Kv1.5 when inhibited by 4-AP (Fig. 22). In contrast, Kv1.2

homomultimeric channels and heteromultimeric channels of Kv1.2/Kv1.5 did demonstrate a positive shift in steady-state activation coincident with 4-AP block (Fig. 22). The shift in steady-state activation coincident with 4-AP block of Kv1.2/Kv1.5 and RPV Kdr channels is consistent with the reported shift of gating currents of mutant, non-conducting *Shaker* channels by 4-AP treatment (Loboda and Armstrong, 2001). In the present experiments steady-state activation was assessed by tail current analysis following 250 msec depolarizing pulses. This provides a measure of steady-state current when the activation of channels is essentially complete and prior to the development of significant inactivation of the Kdr channels. These data provide convincing evidence for the majority of Kdr channels being composed of Kv1.2/Kv1.5 heteromultimers. The steady-state activation properties of Kv1.2/Kv1.5 heteromultimeric channels are also consistent with those reported for RPV Kdr (Table 2).

The heteromultimeric assembly of VSM Kv channels is consistent with previous reports demonstrating that the majority of Kv channel complexes in rat, bovine and human brain are heteromultimeric (Rhodes et al., 1997; Shamotienko et al.. 1997; Coleman et al., 1999). Interestingly, VSM Kdr recorded from coronary and mesenteric myocytes also demonstrates a characteristic shift in the voltage dependence of activation during 4-AP block (Remillard and Leblanc, 1996: Plane and Cole, 2000; Lu et al., 2001). This suggests the predominance of Kv1.2/Kv1.5 heteromultimeric channels in these, and likely other, vascular myocytes.

5.1.4. Role of Kvp subunits in V S M Kv currents.

The expression of modulatory Kvp 1.2, Kvpi.3 and Kvp2.1 proteins was detected in RPV and the Kvpi.2 subunit was demonstrated to associate with Kvl.5 subunits. This is the first demonstration of Kvp subunit protein expression in smooth muscle and their assembly into smooth muscle Kv channel complexes. This result is consistent with a recent report demonstrating the co-IP of Kvl.2, Kvl.4 and Kvl.5 subunits with Kvpi.2 in cardiac tissue (Kuryshev et al., 2001).

RPV KvP subunits likely function to modulate the biophysical properties and membrane trafficking of Kvl.2, Kvl.4 and Kvl.5 channel complexes, as demonstrated in heterologous expression systems (Accili et al., 1997a, 1997b; Xu and Li, 1998; Clement-Chomienne et al., 1999; Manganas and Trimmer, 2000). Kvp 1.2 and Kvp 1.3 subunits evoke fast inactivation of slowly inactivating Kvl.2 and Kvl.5 subunits, which is observed during depolarizing steps to positive potentials (Accili et al., 1997a; Uebele et al., 1998). This rapid inactivation occurs at voltages that are significantly more positive than the physiological range of V S M Em Therefore, the RPV Kto current is not due to the fast inactivation properties of Kvpi subunits, present within channel complexes composed of only Kvl.2 and Kvl.5 pore-forming a subunits. However, it is possible that Kvpi subunits may contribute to channel complexes containing Kvl.4 subunits that underlie the RPV Kto current. Kvl.2/Kvl.5 or Kvl.5 channels demonstrating fast inactivation due to the effects of Kvpi subunits may be present in RPV myocytes, but compose a very minor component of the whole-cell Kv current. A very minor population of these channels, demonstrating fast inactivation at only positive potentials, would not

be observable in RPV myocytes due to the predominance of slowly inactivating Kdr channels.

Further explanations for the demonstrated association of Kvp1.2 subunits with Kv1.5 in RPV despite the lack of fast inactivation at positive voltages of the native Kv whole-cell current are: i) The binding of the inactivation domain of the Kvpi subunit to a N-type inactivation prevention (NIP) domain similar to that documented in Kv1.6 (Roeper et al., 1998), but present in another channel-associated protein, ii) The lack of fast inactivation due to the redox state of Kvpi subunits in RPV, as suggested for Kvpi.1 and KvP3.1 subunits upon mutation of their oxidoreductase catalytic site (Bahring et al., 2001). iii) An interaction of the Kvp subunit amino terminus with cytoskeletal elements of RPV myocytes, as described in COS-1 cells (Nakahira et al., 1998), resulting in the loss of Kvp1-induced fast inactivation. Clearly the functional roles of Kvp subunits are incompletely understood in smooth muscle, as well as in other excitable tissues, and will require further investigation.

5.1.5. Molecular basis of VSM Kdr regulation.

The assembly of Kv1.2 into RPV Kdr channels, as implicated by the co-immunoprecipitation of Kv1.2 with Kv1.5 (Fig. 21) and pharmacological data utilizing 4-AP (Fig. 22) (Kerr et al., 2001), is consistent with the documented potentiation of RPV Kdr by PKA and inhibition by PKC activation (Aiello et al., 1995. 1996, 1998; Clement-Chomienne et al., 1996). Homomultimeric channels of Kv1.2 have demonstrated potentiation of activity following PKA activation (Fig. 25; Huang et al., 1994), and inhibition by PKC via the activation of tyrosine kinase signaling pathways (Huang et al.,

1993; Vogalis et al., 1995; Tsai et al., 1997, 1999). Evidence has also been provided for the modulation of Kv1.2/Kv1.5 heteromultimeric channels by PKC (Vogalis et al, 1995). This study demonstrates a lack of effect of PKA on homomultimeric Kv1.5 channels and a potentiation of Kv1.2 homomultimers in HEK cells. It seems likely that Kv1.2 confers modulation by PKA and PKC to Kv1.2/Kv1.5 heteromultimeric channels present in RPV myocytes. Further experiments will be required to elucidate the role of Kv1.2 in mediating the regulation of native VSM Kdr by PKA and PKC.

It has been suggested that Kvpi subunit-mediated fast inactivation may be regulated by cellular phosphorylation events involving PKA and PKC (Kwak et al, 1999a, 1999b). Interestingly, the rabbit Kvp1.3 subunit lacks the key PKA phosphorylation site (S24) (Fig. 7), present in the human Kvp1.3 subunit, and documented to be responsible for the reduction in fast inactivation due to PKA activity. Consistent with a lack of the PKA phosphorylation site in rabbit Kvpi.3, previous experiments demonstrating regulation of RPV Kdr by signaling pathways involving PKA and PKC activation did not detect changes in the degree of inactivation of RPV Kdr whole-cell currents (Aiello et al., 1995. 1996, 1998; Clement-Chomienne et al.. 1996). Therefore, these data suggest that PKA- and PKC-dependent regulatory mechanisms modulating the extent of Kvpi-mediated fast inactivation are not functional in RPV VSM.

5.1.6. Conclusion: Molecular identity of VSM Kv channels

As outlined in the Introduction, a number of criteria must be met to provide substantial evidence for the contribution of specific channel subunits to a native K^+

channel. Only through the combined use of different, complimentary techniques, which provide a number of lines of evidence, can a firm molecular identification of channel composition be established. This study utilizes this multidisciplinary approach, by providing the basis of Kv subunit expression via determining mRNAs expressed in RPV VSM tissue and confirmation of the presence of proteins encoded by expressed mRNA by immunolabeling of freshly isolated RPV vascular myocytes and Western blotting of RPV proteins. Furthermore, the co-association of expressed subunit proteins was probed by co-immunoprecipitation experiments, providing the first analysis of Kv subunit assembly in smooth muscle. The expression and assembly pattern of Kv subunits was supported by an analysis of the pharmacology, biophysical properties and regulation of recombinant channels. This study provides compelling evidence for a role of expressed channel subunits in a defined assembly pattern.

Specifically, this study demonstrates the expression of Kvl.2, Kvl.4, Kvl.5, KvP1.2, Kvp1.3 and Kvp2.1 proteins as chamiel subunits underlying the 4-AP-sensitive Kv current of RPV vascular myocytes. It also provides the first evidence for the formation of heteromultimeric Kv channels in smooth muscle by the co-immunoprecipitation of Kvl.2, Kvl.5 and Kvl.5, KvP1.2 subunits. It is concluded from this study that the 4-AP-sensitive Kdr component of RPV is largely the result of heteromultimeric channels composed of Kvl.2 and Kvl.5 a subunits and the expression of channels containing Kvl.4 a subunits is responsible for the previously described. A-type, Kto current in these vascular myocytes.

5.2. VSM Kjyj)p channels

5.2.1. Expression pattern of K^TP channel subunits in VSM.

This study provides the first comprehensive screening of the expression pattern of known K^jp channel subunits in VSM and provides evidence for the expression of Kir6.1, Kir6.2, SUR2A and SUR2B mRNA and a lack of SUR1 mRNA in RPV. Very few studies have been performed demonstrating the expression of specific K^jp subunits in smooth muscle. Kir6.1 mRNA expression has been demonstrated in human and mouse aorta (Surah-Narwal et al, 1999; Suzuki et al., 2001). RT-PCR performed on mRNA isolated from mouse colonic smooth muscle myocytes has demonstrated an expression of Kir6.2 and SUR2B mRNA within the myocytes, and a lack of detection of Kir6.1, SUR2A and SUR1 subunits (Koh et al., 1998). In this particular study, mouse colonic myocytes demonstrated the presence of a 27 pS K^jp channel (in symmetrical 140/140 mM K^+ conditions) that demonstrated a loss of activity in 1-0 patches excised into ATP- and ADP-free solution. 1-0 patches of the 27 pS channel were reactivated with the subsequent treatment of the cytoplasmic face of patches with 1 mM ADP and 0.1 mM ATP. These properties are consistent with those of VSM **KNJJP** channels (Zhang and Bolton, 1996; Cole et al., 2000) and Kir6.1/SUR2B channels (Yamada et al., 1997). However, these results are difficult to reconcile due to the expression of only Kir6.2 and SUR2B subunits, which form channels activated in the presence of ATP-free solutions (Quayle et al., 1997; Seino. 1999). As well, the conductance of Kir6.2 channels has been demonstrated to be markedly larger (~ 70-80 pS) than that reported in the study of the mouse colonic channel of 27 pS (Aguilar-Bryan et al., 1998; Seino, 1999). One must

question the functionality of primers utilized for Kir6.1 RT-PCR in this study, as no positive control was shown confirming the ability of the primers to amplify mRNA encoding this subunit. Therefore, from these data it is difficult to conclude that Kir6.1 subunits are not expressed in mouse colonic myocytes.

5.2.2. **Kir6.1 and** SUR2B **subunits underlying K\ j)p channels.**

The dominance of Kir6.1 and SUR2B channel subunits in forming functional V S M KjsjQp channels is demonstrated by the following: i) The expression of Kir6.1 and SUR2B subunits in V S M (Fig. 26) (Surah-Narwal et al, 1999; Suzuki et al., 2001). ii) Similarity of single-channel conductance of **K ^ D P** channels to Kir6.1 channels (~ 35 pS) (Fig. 27), but not to channels formed by the only other known pore-forming subunit Kir6.2 (~ 80 pS). iii) Unique activation of **KNJJP** channels and recombinant Kir6.X/SUR2B channels, but not Kir6.X/SUR2A or Kir6.X/SURl channels, by low [ATP] (<1 mM) and NDPs (Satoh et al., 1998; Cole and Clement-Chomienne, 2000; Matsuoka et al., 2000). iv) Similar pharmacology of K ^ p p and Kir6.1/SUR2B channels with respect to their activation by the K^+ channel opener diazoxide. This K^+ channel opener has been shown to require the unique spliced carboxy terminus of SUR2B. which has also demonstrated an importance for the stimulatory effects of NDPs on channel activity (Yamada et al., 1997; Satoh et al., 1998; Matsuoka et al., 2000). v) PKC-mediated inhibition of Kir6.1/SUR2B channel activity (this study) and V S M KjvjDP channel activity (Cole et al., 2000) by increasing the inter-burst interval. This is in sharp contrast to the demonstrated increases in Kir6.2/SUR2A or Kir6.2/SUR1 channel activity by PKC activation (Light et al.. 2000).

It is possible that Kir6.2 or SUR2A subunits may contribute to RPV K^pp channels. This is favoured by evidence suggesting heteromultimeric assembly of Kir6.1 and Kir6.2 subunits in expression systems (Kono et al, 2000; Cui et al., 2001), and by the detection of these channel transcripts by RT-PCR of RPV mRNA (Fig. 26). On the other hand, the heteromultimeric assembly of these pore-forming subunits has been suggested not to occur *in vivo* (Seharaseyon et al., 2000). In addition, this study detecting Kir6.2 and SUR2A subunits in RPV may reflect the detection of these subunits from contaminating cell types present in the RPV tissue samples from which mRNA was isolated. RPV myocytes exhibit one **K ^ D P** channel population (Beech et al., 1993b; Cole et al., 2000), inconsistent with the multiple channel populations observed upon the heterologous co-expression of Kir6.1 and Kir6.2 subunits (Cui et al., 2001). Nonetheless, if Kir6.2 and SUR2A subunits do contribute to **KNDP** channel complexes in RPV, the properties of the Kir6.1 and SUR2B subunits present within the channel complexes dominate.

5.2.3. Effect of PKC on Kir6.1/SUR2B channel activity.

This study provides the first analysis of the regulation by PKC of **K**^-pp channels composed of Kir6.1/SUR2B. PdBu and the agonist, angiotensin II, lead to a decline in pinacidil-induced Kir6.1/SUR2B channel activity in C-A patches of HEK cells, an inhibitory effect that was suppressed by pre-treatment of cells with the PKC-selective inhibitor chelerythrine. Kir6.1/SUR2B channels in 1-0 patches, which were activated by ADP and ATP included in the bathing solution, demonstrated an inhibition of activity due to treatment with purified PKC. This inhibitory effect of PKC was not observed when

PKC was applied in the presence of its pseudosubstrate inhibitor peptide. PKC(19-31), indicating the enzymatic activity of the purified PKC as the mediator of channel modulation. These results are in contrast to the effect of PKC activation on channels composed of Kir6.2 and SUR2A or SUR1 subunits, the proposed cardiac and pancreatic P-cell **KATP** channels, respectively (Seino, 1999). Both channel combinations demonstrated an enhancement of channel activity by PKC activation, under similar recording conditions (Light et al., 2000). Thus, the inhibition of Kir6.1/SUR2B channels by PKC, identified in this study, provides novel functional evidence that strongly supports this combination of subunits contributing to **KNDP** channels of vascular myocytes.

5.2.4. Mechanism underlying PKC inhibition of Kir6.1/SUR2B channels.

The mechanism of inhibition of Kir6.1/SUR2B channels by PKC activity was investigated by an analysis of burst kinetics. Burst duration and intra-burst open and closed dwell times were found to be unaffected by PdBu. The high level of expression of Kir6.1/SUR2B channels in HEK cells resulted in more than one channel in all patches in this study, which precluded a direct measurement of inter-burst interval. However, a lack of effect of PKC activation on burst duration and intra-burst kinetics provides indirect evidence for an increase in the inter-burst interval as the mechanism underlying the inhibitory effects of PKC on Kir6.1/SUR2B channel activity. A decline in activity with PKC activation due to an increase in the inter-burst interval is consistent with the mechanism identified for PKC channel inhibition of native VSM Kjsjrjp channels. Experiments in which patches containing one native K^pjp channel were obtained.

enabled a direct measurement of inter-burst interval with and without PKC activation (Cole et al, 2000).

It has been proposed that the inter-burst interval of K^jp channels represents a long-lived closed state due to the tight binding of inhibitory ATP to the channel (Takano and Noma, 1993). Inhibition of native VSM Kjsjrjp channels and recombinant Kir6.1/SUR2B channels by PKC caused by an increased inter-burst interval, suggests a PKC-dependent modulation of the interaction of ATP with the channels. It seems likely that PKC phosphorylation results in an increase in the affinity of these channels for ATP, thereby increasing the time ATP remains tightly bound to the channel, i.e. the duration of the inter-burst interval. The effects of PKC on recombinant Kir6.2 channels co-expressed with SUR2A or SUR1 are consistent with this view, as the sensitivity of Kir6.2 channels to ATP was altered by PKC activity (Light et al., 2000). At low [ATP] (<100 pM) the Kir6.2 channel sensitivity to ATP inhibition was increased, resulting in decreased channel activity. In contrast at physiological [ATP] (>100 pM), a decrease in sensitivity to ATP inhibition was observed, causing potentiation of channel activity upon PKC activation. The differing effect of PKC on Kir6.2 channel activity, dependent upon the [ATP], is consistent with results obtained with native cardiac K^TP channels (Light et al., 1995). Furthermore, the phosphorylation site responsible for PKC modulation of Kir6.2 was determined to be T180 of the Kir6.2 pore-forming subunit. T180 is present in the carboxy terminus of Kir6.2 located on the cytoplasmic side of the channel and within a region known to mediate effects of ATP on channel function (Shyng et al., 2000). Mutation of the analogous residue of Kir6.1 (T190) to an alanine or cysteine residue, in

this study, yielded mutant channels with very low channel activity (data not shown). These results demonstrated the importance of the T190 residue in Kir6.1 channel function, but prevented an analysis of the effects of PKC on these mutants.

The inhibitory effect of PKC on Kir6.1/SUR2B channel activity was observed in C-A patches under conditions in which cytoplasmic [ATP] was not controlled. Nonetheless, one may assume mM concentrations of ATP were present due to the inclusion of glucose in recording solutions (Nelson and Quayle, 1995; Gollasch et al., 2000). 1-0 patches of KM.1/SUR2B channels were also utilized in this study under conditions of defined [ATP] (0.5 mM). An inhibition of Kir6.1/SUR2B channel activity by PKC was observed at [ATP] at which Kir6.2/SUR2A channels demonstrated a potentiation of channel activity. The contrasting effects of PKC activity on Kir6.1/SUR2B and Kir6.2/SUR2A channels at the same [ATP] is consistent with the markedly different sensitivities to ATP displayed by these two pore-forming channel subunits. Kir6.1/SUR2B channels are inactive when excised into ATP/ADP-free solutions, as demonstrated by their bell-shaped ATP sensitivity curve. Activation of Kir6.1/SUR2B channels is observed at [ATP] of 0.1 to 1 mM followed by an inhibition of channel activity at [ATP] above 1 mM (Satoh et al., 1998). Kir6.2/SUR2A and Kir6.2/SUR1 channels on the other hand demonstrate robust activity in the absence of ATP and are inhibited in a concentration-dependent manner with increasing [ATP] (Light et al., 2000).

5.2.5. Role of Kir6.1/SUR2B channels in V S M K ^ T P whole-cell currents.

A number of different smooth muscle K ^ T P single-channel conductance values have been reported in the literature, which in general terms can be separated into two different groups; large and small conductance channels (reviewed by Quayle et al, 1997). The larger conductance channels (>100 pS) have been suggested to be due to the misidentification of smooth muscle B K ^ $_a$ channels (Zhang and Bolton, 1995; Cole and Clement-Chomienne, 2000). This interpretation is based on the lack of cloning, to date, of a **K-ATP** channel pore-forming subunit of this magnitude in conductance. In addition, some studies reported a voltage or C a ^ $^+$ sensitivity of the large conductance channel. The presence of two distinct K ^ j P channels in rat portal vein has been demonstrated, one identified as a ~ 35 pS KfvjTjp channel and the second as a cardiac-like, ~ 80 pS channel (Zhang and Bolton, 1996; Cole et al., 2000). In some studies of V S M , such as those from RPV and mesenteric arteries, the K ^ ^ p channel was the sole K ^ T P conductance detected (Beech et al., 1993; Zhang and Bolton, 1995; Cole et al., 2000). Significantly, the larger ~ 80 pS channel of rat portal vein was determined to be unaffected by P K C activation, in contrast to the inhibition observed for the Kfyfpjp channel (Cole et al., 2000). This evidence supports a dominant role of Kjsjpjp channels as mediators of smooth muscle whole-cell K ^ T P currents that have demonstrated inhibitory modulation by agonists leading to P K C activation. Moreover, the indication of Kir6.1/SUR2B channels as the molecular entities underlying V S M Kj^rjp channels, and the ability of P K C activation to inhibit Kir6.1/SUR2B channels, suggests a dominant role of Kir6.1/SUR2B

channels in smooth muscle K ^ **T P** whole-cell currents (Bonev and Nelson, 1993, 1996; Kleppisch and Nelson, 1995). Consistent with this view, whole-cell smooth muscle K.ATP current recordings that demonstrated modulation by PKC activation were performed in the presence of NDP and sub-millimolar ATP in the pipette solution. These are conditions supporting the activity of Kjsjpjp channels, but not cardiac-like K ^ **T P** channels.

5.2.6. Conclusion: Molecular identity of VSM KJ^DP channels

This study demonstrates the expression of mRNA encoding K^jp subunits Kir6.1, Kir6.2, SUR2A and SUR2B, but not SUR1 in RPV VSM. Moreover, it provides the first functional evidence for Kir6.1/SUR2B subunits underlying native VSM **KNDP** channels, in the form of a similar regulation of Kir6.1/SUR2B channels and VSM **KNDP** channels by PKC activity. Kir6.1/SUR2B channels were expressed in HEK cells and demonstrated inhibition of activity by angiotensin II and activation of PKC. PKC-mediated inhibition of Kir6.1/SUR2B channel activity was demonstrated to occur by a mechanism that involves an increased inter-burst interval of channel gating, consistent with the mechanism underlying the inhibition of native K]sjp)p channels by PKC activation. The demonstrated expression of K^fjjjp channels in a number of VSM myocytes and the ubiquity of PKC-mediated modulation of whole-cell VSM K^jp currents by agonists suggests that Kir6.1/SUR2B channels play a central role as contributors to VSM K^-pp whole-cell currents.

5.3. Conclusion

This study provides strong evidence for the identification of K^+ channel subunits contributing to Kv and K ^ p channel complexes of PvPV V S M. This is achieved by the use of complementary molecular biological, biochemical and electrophysiological techniques that provide several lines of evidence indicating a role for individual subunits. This approach demonstrates the expression of channel subunits at the mRNA and protein levels, and provides functional data by the pharmacological manipulation of channel clones expressed in a heterologous expression system.

CHAPTER 6: FUTURE DIRECTIONS

6.1. Molecular identification of vascular smooth muscle Kv channels.

6.1.1. 4-AP-sensitive Kv component of vascular smooth muscle.

Although this study provides strong evidence for a major role of Kv1 channels in VSM, Kv2 and Kv3 family members have also been implicated in the control of other VSMs. For example, channels composed of Kv2 and Kv3 subunits were implicated as candidates in mediating hypoxic pulmonary vasoconstriction (Patel et al., 1997; Osipenko et al., 2000). Therefore, an analysis of RPV expression of other Kv family members is warranted. A similar approach to that utilized in this study involving RT-PCR and immunolabeling for proteins detected at the mRNA level is required. Kv2, Kv3, and possibly Kv4 channels, may be expressed in RPV and may underlie a minor portion of the RPV whole-cell Kv current. However, as stated, these channels are expected to play a minor role in the control of RPV Em, based upon the dominant contribution of 4-AP-sensitive Kv1 channels.

A comprehensive analysis of the expression pattern of Kv subunits in other vascular beds, by an analogous approach to that applied here for RPV, is also of great importance. One can speculate that differences in the expression of Kv subunits in different vessels, such as coronary, cerebral and mesenteric arteries, may provide a basis for the observed differences in Kv whole-cell current biophysical properties (Table 1). One would hypothesize that the expression pattern in other vascular smooth muscles is similar to that in RPV, but not identical.

6.1.2. 4-AP-insensitive Kv component of vascular smooth muscle.

Significantly, ~ 70% of the Kdr current in RPV, coronary and mesenteric vascular myocytes is inhibited by 4-AP (Remillard and Leblanc, 1996; Clement-Chomienne et al., 1999; Lu et al., 2001). This suggests a role for 4-AP-insensitive, Kv-like channels contributing to ~ 30% of Kdr in these vascular myocytes. To determine the molecular identity of the 4-AP-insensitive Kdr current of RPV, an analysis of the sensitivity of this residual current to different ion channel blockers is required. It is presumed that the 4-AP-insensitive Kv current is carried by K^+ selective channels as preliminary experiments conducted with the inclusion in the whole-cell pipette solution of 10 mM tetraethylammonium ion, a general K^+ channel blocker, demonstrated a complete block of both the 4-AP-sensitive and -insensitive RPV Kdr current components. A large family of twin pore domain K^+ channels has recently been discovered in conjunction with the sequencing of numerous genomes (reviewed by Lesage and Lazdunski, 2000). Members of this novel family of channels may provide the basis of this 4-AP-resistant, Kv-like current in VSM.

6.1.3. Reconstitution of the modulation of native Kdr current by vasoactive agonists utilizing the heterologous expression of Kv subunit clones.

Knowledge of the molecular constituents of VSM Kv channels is of critical importance in designing pharmacological tools to modulate K^+ channel function in treatment of vascular disease. In addition, an understanding of the molecular mechanisms responsible for modulating VSM Kv channels by vasoactive agonists can provide further clinical targets for the regulation of K^+ channel activity in control of vascular tone.

Vasoactive agonists signaling through PKA and PKC activation result in a potentiation and a reduction of VSM Kv current, respectively. These mechanisms are potential targets for a modulation of Kv current and Em by therapeutic agents.

This study demonstrates that the expression of cloned Kv1.2 and Kv1.5 channels in a mammalian HEK cell line provides for the regulation by PKA activation of Kv1.2 homomultimeric channels, but not Kv1.5 homomultimeric channels. Preliminary experiments were performed investigating the effects of PKA activation on heteromultimeric channels formed by Kv1.2 and Kv1.5 subunits. The expression of the tandem Kv1.5-Kv1.2 construct, and co-expression of Kv1.2 and Kv1.5 channel clones, failed to demonstrate currents regulated by PKA activation. Further investigation into the PKA modulation of heteromultimeric channels composed of Kv1.2 and Kv1.5 subunits will be required. Perhaps the assembly ratio of Kv1.2 and Kv1.5 subunits, with or without other associated proteins, such as Kvp subunits, is important for their modulation by PKA. This can be addressed by the preparation of tandem constructs yielding expressed channels with differing ratios of Kv1.2 and Kv1.5 assembly. Similar experiments must be conducted to examine the effect of PKC on Kv1.2/Kv1.5 heteromultimeric channels. Evidence in the literature supports an effect of PKC on modulation of these two cloned channels, but this has not been studied using a mammalian system.

6.1.4. Role of Kvp subunits in native VSM Kv channels.

An emerging field of study probes the role of KvP subunits in Kv channel function. An effect of KvP subunits on channel gating and trafficking of channel complexes to the membrane has been well documented. Recently, the interaction of Kvp

subunits with elements of the cytoskeleton (Nakahira et al., 1996), and the ability of KvP subunits to sense changes in cellular redox potential due to inherent oxidoreductase properties (Gulbis et al., 1999; Bahring et al., 2001), have been documented. A role of redox potential in the control of native V S M Kv channels is implicated by the hypoxic constriction of pulmonary vessels. KvP subunits may, therefore, play an important role in coupling the redox state of smooth muscle tissue to Kv channel function, resulting in modulation of vascular tone. Experiments employing agents that modulate the assembly of cytoskeletal elements and cellular redox potential will help determine if these factors are important for the regulation of vascular tone via the modulation of Kv channel function.

6.1.5. Knock-down of native Kv channel function.

Once the molecular basis of Kv channels in V S M is defined, the use of anti-sense strategies, genetic knock-outs and dominant-negative constructs specific for Kv subunits can be utilized in an analysis of the importance of expressed Kv subunits in the control of vascular reactivity. Viral gene transfer has been utilized recently to express dominant-negative channel constructs, resulting in the suppression of native cardiac K^+ channel currents (Guo et al., 2000; Suzuki et al., 2001). Experiments utilizing these approaches for analysis of changes in Em and myogenic reactivity of intact vessels can provide evidence for a role of specific channel subunits in the control of vascular tone.

6.2. Molecular identification of VSM K^TP channels.

6.2.1. Expression pattern and assembly of VSM K^TP channel subunits.

A combined molecular and biochemical approach is required in order to demonstrate the expression of individual K^TP subunits within vascular myocytes. Subunit-specific RT-PCR utilizing mRNA isolated from dissociated RPV myocytes is required in order to determine if the detected expression of Kir and SUR subunits from mRNA isolated from RPV tissue is due to the expression of these subunits within the vascular myocytes. In addition, the development of subunit-specific antibodies for Western blotting, immunocytochemistry of freshly isolated myocytes and IP of K^jp channel complexes from VSM is required in order to demonstrate the expression of specific subunit proteins and to determine the assembly pattern of subunits into VSM ^ATP channels. IP experiments would also determine whether multiple pore-forming subunits, such as Kir6.1 and Kir6.2, co-assemble to form heteromultimeric K^jp channels in VSM.

6.2.2. Regulation of heteromultimeric K^TP channels by PKC.

It has been demonstrated that Kir6.2/SUR2A and Kir6.2/SUR1 channels are activated by PKC at physiological [ATP] while Kir6.1/SUR2B channels demonstrate an inhibition under similar conditions. The effects of PKC activation on Kir6.2/SUR2B channels and heteromultimeric K^-pp channels composed of Kir6.1/ Kir6.2 and SUR2B subunits would also shed light on the potential role of the Kir6.2 subunits in contributing to VSM Kfy[p)p channels.

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