ASPECTS OF AUTOREGULATION

THE EFFECTS OF MITOGEN-ACTIVATED PROTEIN KINASE AND

GAP JUNCTION COMMUNICATION INHIBITION ON MYOGENIC

TONE

by

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ABSTRACT

AUTOREGULATION of blood flow in small arteries and arterioles is a combination of myogenic, neural, humoral and metabolic effects. The interaction between these effects allows capillary blood flow to remain relatively constant in spite of changes in blood pressure. And, myogenic or pressure-induced tone significantly contributes to the overall contractile state of many vascular beds for example, in the coronary, mesenteric, skeletal muscle and cerebral circulations. Cytosolic calcium concentration is the primary factor that governs the contractility during the myogenic response. However, several cellular events can modulate the reactivity of smooth muscle cells. Two such processes include the mitogen-activated protein (MAP) kinase and intercellular communication mediated via gap junctions.

Two inhibitors of MAP kinase, U0126 and PD98059, were used to determine the effects of MAP kinase inhibition on myogenic tone in the rat middle cerebral artery. In addition, their inhibitory effects on vasopressin- and depolarization-induced constrictions were determined. PD98059 was nonselective and partially inhibited the three forms of vasoconstriction. U0126 (at 10μM) selectively inhibited myogenic tone and agonist-induced tone but not depolarization-induced tone. Furthermore, indolactam, an activator of protein kinase C, was not affected at this concentration. These results suggest that MAP kinase may be involved in the regulation of myogenic and agonist-induced tone.

Two inhibitors of gap junction coupling, heptanol and α-glycyrrhetinic acid (α-GA) were used to determine the role of cell-to-cell communication in myogenic tone. Both inhibitors exhibited similar trends in inhibiting myogenic and vasopressin-induced tone. However, heptanol also inhibited depolarization-induced tone but not α-GA indicating that heptanol may inhibit voltage-gated Ca^{2+} channels. These results suggest that myogenic tone may be mediated through a similar signaling cascade as the receptor-mediated vasopressin response. Also, the selectivity of α-GA suggests that a significant proportion of smooth muscle cells are insensitive to agonist or pressure stimulation and require gap junction-coupled cells for activation.

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1. INTRODUCTION

1.1. PREFACE

The myogenic response is an intrinsic property of small resistance blood vessels that originates in the smooth muscle and allows them to constrict in response to increases in transmural pressure. Conversely, vasodilation accompanies a decrease in transmural pressure. Myogenic control of vessel diameter is one factor of several including pulsatility of blood pressure, neurotransmitter and endothelial influences which when taken together form an intimate and coordinated control of blood flow. Although the relative contribution of these mechanisms varies from different vascular beds, the role of myogenic reactivity in, for example, the mesenteric, skeletal muscle, coronary, and cerebral circulation is significant. The resultant blood flow in the capillaries is not uniform and depends chiefly on the contractile state of arterioles and the immediately preceding small arteries. While cytosolic concentrations of free Ca^{2+} is the primary signal for vasoconstriction, the mechanism(s) underlying the sensitivity of vascular smooth muscle to this signal is hypothetical. Moreover, the collective response of each individual smooth muscle cell to pressure or agonist may depend upon cell-to-cell communication mediated via gap junctions and results in a concerted constriction.

This thesis investigates the *in vitro* role of mitogen-activated protein (MAP) kinase in modulating the myogenic response to pressure in rat cerebral small arteries. In addition, the role of intercellular communication in these vessels is evaluated.

1.2 MYOGENIC TONE

1.2.1. INTRODUCTION

The first report of pressure-induced responses in blood vessels was by Bayliss in 1902. He described the vasomotion of the canine carotid artery caused by an elevation of internal pressure as a "myogenic response". Since then, the terms, myogenic response, tone, and reactivity have all been used to describe the inherent property of small arteries and arterioles to constrict in response to increases in the transmural pressure, and conversely, these terms include the resulting vasorelaxation when pressure is lowered. It is this dynamic regulation of precapillary vessel diameter that governs the resistance to flow. Consequently, regional vascular perfusion is maintained in spite of variations in blood pressure.

Myogenic tone in small vessels is, by itself, only a component of autoregulation; metabolic, humoral and neural factors may also contribute to the overall resistance in a given vascular bed. However, it has been suggested that myogenic control contributes significantly to autoregulation in tissues such as in the mesenteric, skeletal, cerebral, renal and coronary circulations (for reviews, Johnson, 1989, Muller et al. 1996, Faraci et al. 1989, Roman and Harder, 1993, Bevan and Laher, 1991). This integration of intrinsic and extrinsic autoregulatory factors mitigates the ability to interpret the myogenic responses in *in vivo* microcirculatory and *ex vivo* whole organ perfusion models. Therefore, the study of myogenic reactivity in small blood vessels has been mostly limited to in vitro isometric ring preparations and more recently the pressure myograph (Halpern et al. 1984).

1.2.2. PRESSURE MYOGRAPHY

The pressure myograph is essentially a tissue bath where two axially aligned and apposed glass microcannulae (tip diameter = 30 – 70 µm) are immersed and onto which the ends of a tissue segment are fixed. When the distal cannula is clamped, the intraluminal pressure can be changed using either a pressure-servo controlled peristaltic pump or a solution-filled graduated column connected in series. Intraluminal pressure can also be measured using a flow-though pressure transducer. And, since the extraluminal pressure is negligible, the intraluminal pressure can be used as a measure of the effective transmural pressure. Vessel diameter is most commonly measured using a video edge-detection system. This system has the advantage of being more physiological compared to the wire myograph.

1.2.3. ROLE OF ENDOTHELIUM

The myogenic response is modulated but not dependent on the endothelium. Early studies on the role of the endothelium were contradictory in that the removal of the endothelial layer either altered or had no effect on the myogenic response (for review, Meininger and Davis, 1992). Critical analysis of these studies revealed a methodological basis for this discrepancy; data supporting the role of the endothelium in the myogenic response were obtained from studies that chemically removed the endothelium in contrast to mechanical methods where the participation

of the endothelium was not observed. However, the endothelium of cerebral arteries appears to be involved in myogenic response, at least in the cat and rat. Perfusate from the endothelium-intact, myogenically active cat cerebral artery restored the myogenic reactivity in an endothelium-denuded artery (Harder et al. 1989). Therefore, in cat cerebral arteries, the endothelium may have a critical role in pressure-induced vasoconstrictions. Removal of the endothelium in the rat cerebral artery caused an increase in the level of myogenic tone compared to denuded vessels, and this effect was attributed to the basal release of nitric oxide from the endothelium of the intact vessels (Zimmermann et al. 1997).

1.2.4. CELLULAR MECHANISMS: RECEPTORS AND MEMBRANE DEPOLARIZATION

Numerous studies have demonstrated that smooth muscle cell membrane depolarization coincides with an increase in intraluminal pressure (Harder, 1984, Harder et al. 1985, Harder et al. 1987, Smeda and Daniel, 1988, Knot and Nelson, 1995 and 1998, Wesselman et al. 1997). This event is always accompanied by vasoconstriction. Several hypotheses have been proposed to account for the pressure-induced membrane depolarization.

1.2.4.1. Stretch-Activated Channels

Evidence for the presence of stretch-activated nonselective cation channels in

vascular smooth muscle cells has been found (Davis *et al.* 1992, Setoguchi *et al.* 1997). Stretch of single smooth muscle cells from the porcine coronary artery activated nonselective cation channels that preferentially passed Na^+ cations over K^+ and Ca^{2+} ions and subsequently produced a membrane depolarization (Davis *et al.* 1992). These channels were inhibited by extracellular Ca^{2+} (IC_{50} = 0.9 mM) and gadolinium (IC_{50} = 14 μM). However, other studies have shown that voltage-gated L-type Ca^{2+} channels (VGCC) in the rat cerebral artery (McCarron *et al.* 1997) and calcium-activated potassium (K_{Ca}) channels in the rabbit mesenteric (Dopico *et al.* 1994) and pulmonary arteries (Kirber *et al.* 1992) may also be activated by stretch. Furthermore, membrane depolarization remains elevated in spite of the decreased wall tension (reduced vessel diameter) characteristic of myogenic tone. The signal for stretch-operated channels therefore may not be simply wall tension. Moreover, the myogenic response in rat skeletal, mesenteric (Watanabe et al. 1996) and cerebral arteries (Nelson et al. 1997) is preserved in the absence of extracellular sodium which contradicts the earlier finding that Na^+ ions are the main charge carriers through stretch-activated channels. Therefore, the presence and role of these channels remains to be verified since no specific inhibitor exists and the experimental conditions used to examine them may not mimic the actual conditions that initiate the myogenic response.

1.2.4.2. Integrins

Integrins are a class of membrane-spanning heterodimeric proteins that are hypothesized to be the wall tension sensor in vascular smooth muscle cells (SMC). Recent studies have demonstrated that these proteins can transduce mechanical forces across the cell membrane (Wang et al. 1993) and initiate changes in intracellular pH and $[Ca^{2+}]_i$ (Sjaastad and Nelson, 1997). Therefore, while acting as mediators of cell adhesion, integrins can also transduce biochemical signals across the cell membrane. Integrin-binding peptides containing the integrin-specific arginine-glycine-aspartate (RGD) peptide sequence was shown to inhibit myogenic tone in skeletal muscle arterioles (Mogford et al, 1996). And, in patch-clamp studies, the Ca^{2+} current was inhibited by RGD peptides specific for the α_V-β_3-integrin and potentiated when the α_5-β_1-integrin was bound by the insoluble α_5-antibody (Wu et al. 1997). Therefore, these proteins appear to have a role in the modulation of $[Ca^{2+}]_i$ during the myogenic response. However, whether integrins are the putative stretch-receptors that initiate the signaling cascade for myogenic tone and if VGCC or other channels are modulated by integrins remain to be determined.

1.2.4.3. K^+ Channels

Inhibition of K^+ channels may also cause membrane depolarization. Of the 5 major types of K^+ channels present in vascular smooth muscle cells, only the voltage-dependent (K_V) and Ca^{2+}-activated K^+ channels appear to have a role in the myogenic response. K_V channel-mediated hyperpolarization probably acts as a

negative feedback to membrane depolarization (Nelson et al. 1990) and therefore may be a secondary event to depolarization (Knot and Nelson, 1995). K_{Ca} channels may also counteract membrane depolarization because of their characteristic sensitivity to increased $[Ca^{2+}]_i$ and voltage dependence. The source of Ca^{2+} may come from VGCC or from spontaneous transient release of Ca^{2+} from the sarcoplasmic reticulum or sparks, the frequency of which increases during SMC activation (Nelson et al. 1995). Harder et al. (1995) proposed that the production of an endogenous inhibitor of the large conductance K_{Ca} (BK) channels might be responsible for the membrane depolarization. 20-Hydroxyeicosantrienoic acid (20-HETE), a metabolite of arachidonic acid (AA) produced through cytochrome P-450 β-hydroxylation was identified as a possible tonic inhibitor of BK channels. Nanomolar levels of this inhibitor were released from cerebral and renal arterioles in a pressure-dependent manner (Harder et al. 1994 and 1997, Zou et al. 1996). The application of 20-HETE to isolated canine renal artery SMC produced a contraction and membrane depolarization (Ma et al. 1993). Additionally, other modulators of BK channel activity such as protein kinases A, G and C have been reported (Sadoshima et al. 1988, Robertson et al. 1993, Minami et al. 1993, Schubert and Gagov, 1997). However, the multitude of modulators, the limitations of patch-clamp studies and the lack of tonic activity in some blood vessels (Jackson and Blair, 1998, Liu et al. 1998, Paterno et al. 1996, Yuan, 1995) prevent conclusive evidence for the role of K_{Ca} channels in myogenic tone.

1.2.4.4. Chloride Channels

Chloride channels have also been hypothesized to mediate membrane depolarization. A volume-regulated chloride channel in vascular smooth muscle cells has recently been reported (Yamazaki et al. 1998). Chloride channel blockers inhibited myogenic tone as pressure was increased (Nelson et al. 1997), albeit one of the inhibitors used was a potent Ca^{2+} channel blocker (Doughty et al. 1998). Therefore, the lack of a specific inhibitor of these channels prohibits a definitive answer to this hypothesis.

1.2.4.5. Voltage-Gated Ca^{2+} Channels

The possibility that membrane depolarization may be caused by the activation of VGCC has been considered. Inhibition of Ca^{2+} influx with Ca^{2+} channel blockers abolished myogenic tone in the rat (Knot and Nelson, 1998) and rabbit cerebral arteries (Knot and Nelson, 1995) but did not affect membrane depolarization. Furthermore, after clamping the membrane potential with a high-potassium solution, the myogenic responses in the rat cerebral was reduced but still present (McCarron et al. 1997). These data suggest that the influx of Ca^{2+} via VGCC is not responsible for the membrane depolarization and that intracellular $[Ca^{2+}]$ is an obligatory factor in the myogenic response.

1.2.5. CELLULAR MECHANISMS: INTRACELLULAR SECOND

MESSENGERS

1.2.5.1.   Intracellular Ca^{2+}

Numerous studies have established that in myogenically active vessels, $[Ca^{2+}]_i$

increases as transmural pressure increase (Zou et al. 1995, Yip and Marsh, 1996,

Karibe et al. 1997, VanBavel et al. 1998, Boltz and Pohl, 1996, D'Angelo et al.

1997, Knot and Nelson, 1997). There is much evidence for the extracellular space

as the source of Ca^{2+} for myogenic contraction. In addition to the studies discussed

above, other investigators have shown that the removal of extracellular Ca^{2+}

abolished the myogenic response in a variety of vessels including the rat cerebral

(Osol and Halpern, 1985), saphenous (Berczi et al. 1992), coronary (Wellman et al.

1996), cremaster (Hill and Meininger, 1994), skeletal (Fredricks et al. 1994), porcine

mesenteric (Reber and Nowicki, 1998), canine renal (Kauser et al. 1991), and

human cerebral arteries (Wallis et al. 1996, Thorintrescases et al. 1997). The

contribution of Ca^{2+} to the myogenic response from intracellular stores has been

investigated primarily with inhibitors of the sarcoplasmic reticulum Ca^{2+} ATPase

(SERCA) such as ryanodine (McCarron et al. 1997, Watanabe et al. 1994).

Inhibition of SERCA with ryanodine did not increase the amplitude of constriction in

rat skeletal or cerebral arteries (30 to 70 mmHg and 40 to 100 mmHg, respectively),

but reduced the rate of development of myogenic tone. Therefore, although the

sarcoplasmic reticulum does not directly contribute to the rise in $[Ca^{2+}]_i$ necessary

for the myogenic response, it may be involved in the modulation of the development

of tone.

1.2.5.2.   *G Proteins and Phospholipase C*

G proteins are activated in coronary smooth muscle cells in response to mechanical stimulation (Wiersbitzky et al. 1994); this finding supports the hypothesis that phospholipase C (PLC) is involved in the mechanotransduction processes of resistance arteries. In addition to the trimeric G proteins associated with typical membrane-bound receptors, small molecular weight monomeric G proteins such as Ras, Rho and Raf have may also be activated. These small-molecular weight G proteins have also been implicated in having a role in the myogenic response (discussed below). Sadoshima and Isumo (1993) have demonstrated that GTP binding of $p21^{ras}$ (Ras) occurs within 1 min of applying a mechanical stretch to cultured neonatal cardiomyocytes. Studies on the involvement of PLC have been limited to the use of U73122, a steroidal inhibitor of PLC and the measurement of the breakdown products of polyphosphoinositides. The inhibition of PLC abolished myogenic responses in cerebral arterioles (Osol et al. 1993) and attenuated pressure-induced vasoconstrictions in afferent arterioles (Inscho et al. 1998). In addition, concentrations of inositol trisphosphate (IP_3) and diacylglycerol (DAG) have been shown to increase 90 s and 15 min following the initial pressure stimulus in dog renal arteries (Narayanan et al. 1994). The production of IP_3 may contribute to the rise in $[Ca^{2+}]_i$ by releasing stored Ca^{2+} from the sarcoplasmic reticulum (SR),

and DAG can directly activate protein kinase C (PKC).

1.2.5.3.   Protein Kinase C

PKC activation is implicated in the Ca^{2+} sensitization of the contractile proteins during the myogenic response. Inhibition of PKC with inhibitors such as calphostin C reduces myogenic tone in human coronary arteries (Miller et al. 1997) and cerebral arterioles (Watanabe et al. 1993) without affecting $[Ca^{2+}]_i$ (Karibe et al. 1997). Also, PKC activators such as phorbol esters and indolactam can enhance myogenic reactivity (Hill et al. 1990, Osol et al. 1991). And, subconstrictor concentrations of agonists that activate PKC have been shown to augment the myogenic response (Kirton and Loutzenhiser, 1998, Lui et al. 1993). However, a definitive role of PKC in the pressure-induced response remains to be established because of the lack of specific inhibitors of PKC and the possibility that the 11 identified isozymes may be differentially expressed in different tissues (Hofmann, 1997). For example, both H-7 and staurospaurine compete at the same ATP binding site that is highly conserved between various protein kinases (Ruegg and Burgess, 1989). Moreover, the phosphorylation targets for PKC and its activity during the myogenic response have yet to be fully elucidated. For example, PKC activation has been reported to modulate K_V channel (Kirton and Loutzenhiser, 1998) and VGCC activity (Fish et al. 1988, Galizzi et al. 1987, Hill et al. 1996).

1.2.5.4. Other Protein Kinases

The pressure-induced elevation in $[Ca^{2+}]_i$ is associated with an increased phosphorylation of the 20-kDa regulatory myosin light chain (MLC) in the rat cremaster arterioles (Zou et al. 1995), and the inhibition of MLC kinase dependent phosphorylation with a selective inhibitor ablated the myogenic response to pressure in spite of normal increases in $[Ca^{2+}]_i$. However, 20-kDa Regulatory MLC phosphorylation was also proportionally greater at higher pressures (120-150 mmHg) than at lower pressures (30-70 mmHg). Other investigators have reported inconsistencies in $[Ca^{2+}]_i$ and diameter responses (D'Angelo et al. 1997) suggesting a calcium-independent mechanism for regulating the myogenic response by changing the calcium sensitivity of the contractile machinery. Indeed, when the $[Ca^{2+}]_i$ was clamped in α-toxin-permeabilized rat cerebral arteries no myogenic response was observed over a physiological range of 1 nM to 60 μM (McCarron et al. 1997). It should be noted however, that this study did not find pressure-induced constriction regardless of the calcium concentration but the modulation of Ca^{2+} sensitivity may depend on an intact plasma membrane system. In rat mesenteric small arteries, changes in $[Ca^{2+}]_i$ during the myogenic response were small relative to the $[Ca^{2+}]_i$ required for a comparable constriction elicited with high extracellular potassium (VanBavel et al. 1998). Thus, evidence for active alterations of Ca^{2+} sensitivity in resistance arteries exists (Hill et al. 1996, Gokina et al. 1998), but the subcellular mediator(s) of this event remains unclear.

1.3. MITOGEN-ACTIVATED PROTEIN KINASE

1.3.1. INTRODUCTION

The mechanical stress on vascular SMC causes a number of cellular processes including cell differentiation and vascular remodeling during chronic overt stretch, which results in adaptive alterations of vessel shape and composition. Acute changes in stretch or shear stress results in adjustments in vessel diameter mediated by the release of vasoactive agonists or changes in myogenic tone. For instance, experimental hypertension is accompanied by increased wall thickness resulting from SMC hyperplasia in resistance arteries and arterioles and hypertrophy in conductance arteries (Wolinsky, 1970, Owens et al. 1988). Likewise, reduced mechanical strain translates into vessel atrophy. Vessels placed in conditions of abnormally low intraluminal pressure (10 mmHg) over 3 to 6 days showed decreased content of smooth muscle marker proteins h-caldesmon and filamin in spite of the presence of fetal calf serum, a known culture medium mitogen. In comparison, no marker protein loss was observed in aortic segments kept at a physiological pressure (80 mmHg) (Birukov et al. 1998). Additionally, cyclic stretching of cultured vascular SMC prevented the decrease in expression of smooth muscle myosin heavy chains and myosin light chain kinase (Smith et al. 1995) and augmented myosin heavy chain SM-1 and SM-2 protein content and decreased nonmuscle myosin-A relative to static smooth muscle cell cultures (Reusch et al. 1996). Therefore, stretch is a critical stimulus in SMC cytoskeletal

regulation. The MAP kinase cascade is a major pathway through which signals coming from growth factors and mechanical strain are transduced into gene expression and protein synthesis. MAP kinases are also implicated in thin filament contractile protein regulation through control of the phosphorylation state of the actin and myosin binding protein caldesmon (Adam and Hathaway, 1993).

1.3.2. MAP KINASES: PARALLEL CASCADES

The MAP kinase cascade actually consists of three parallel pathways that respond to different stimuli and instigate distinct cellular responses. The first pathway is the archetypical extracellular signal-regulated kinases (ERK) cascade that is activated by Raf and is present in two isoforms, ERK 1 and 2. This pathway leads to the activation of regulatory proteins in both the cytoplasm and nucleus (Seger and Krebs, 1995). The second branch of the MAP kinase family phosphorylates the amino terminal of the transcription factor c-*jun* (JNK) and is termed stress-activated protein kinases (SAPK) because they are activated by stimuli such as UV light, heat chock, hypoxia or high osmolarity (Eppert et al. 1996, Zhang et al. 1996). The third branch of the MAP kinase family comprises p38, also activated by osmotic stress (Gimbone et al. 1997). The discussion of this thesis will focus mainly on the ERK cascade in cardiovascular smooth muscle with some reference to the other MAP kinase pathways.

1.3.3. ERK KINASES IN THE VASCULATURE

ERK 1/2 kinases are serine-threonine kinases with molecular masses of 42 and 44 kDa, respectively (Force and Bonventre, 1998) and are activated through stimulation of receptor tyrosine kinases such as insulin receptors or G protein-coupled receptors such as thrombin receptors. Involved is the sequential phosphorylation and activation of c-Raf (or MEK kinase and MAP kinase kinase kinase), MEK (or MAP kinase kinase) and finally MAP kinase (Seger and Krebs, 1995). Initiation of the cascade results in the dual phosphorylation of the tyrosine and threonine residues on ERK 1/2 by MEK and is prerequisite for MAP kinase activation (Anderson et al. 1990, Her et al. 1993, Cobb et al. 1995). Currently, there are only two specific inhibitors of MEK kinases, PD98059 and U0126 (Figure 1.1). High intraluminal pressure in aortic organ culture induces a biphasic ERK 1/2 stimulation, characterized by a transient peak in activity followed by an elevated and sustained activation (Birukov et al. 1997). *In vivo*, ERK 1/2 is transiently activated by acute hypertension (Xu et al. 1996) and by vessel wall injury with a balloon catheter (Lille et al. 1997, Pyles et al. 1997). Subsequently, ERK phosphorylates a number of molecules including the cytosolic 10/90 kDa ribosomal S6 kinase (RSK) (Chung et al. 1991), c-Raf (Lee et al. 1992), EGF receptor (Pillay and Sharma, 1995), cytosolic phospholipase A_2 (Lin et al. 1993), and myelin basic protein (Ahn et al. 1991), as well as the transcription factors c-*myc*, C-*jun*, c-*fos* and p62[TCF] (elk-1) (Davis, 1993, Karin, 1995, Janknecht et al. 1993). Therefore, MAP kinase activation may lead to regulation of up- and downstream events. The activation of the transcription factors requires the translocation of MAPK from the cytosol to the

nucleus following its phosphorylation (Gonzalez et al. 1993, Seth, 1992, Sanghera, 1992, Khalil et al. 1995).

1.3.4. ERK KINASE ACTIVATION

Tyrosine phosphorylation is hypothesized to be the link between stretch and smooth muscle contraction (Disalvo et al. 1994, Disalvo et al. 1993, Hollenberg, 1994). Consistent with this hypothesis is the finding that smooth muscle has tyrosine kinase (pp60^{c-src} or c-Src) activity 500 – 700 times that of either skeletal of cardiac muscle (Disalvo et al. 1988). The proposed chain of events leading from stretch to MAP kinase activation begins with integrin activation. Upon endothelial shear stress activation, $\alpha_V\beta_1$ integrins are recruited into clusters and cytoskeletal proteins such as talin, paxillin, vinculin and vinculin-bound α-actinin (Thoumine et al. 1995, Otey et al.1990, Horwitz et al., 1986, Burridge and Mangeat, 1984) aggregate with integrins to form focal adhesions. A focal adhesion kinase (FAK) protein is subsequently phosphorylated and activated by c-Src, which allows the association of a membrane-bound growth factor-related binding protein (Grb2) (Schlaepfer and Hunter, 1997). An intermediary protein coupled to Grb2 termed mSos is then brought into contact with the membrane associated G protein, Ras (Li et al. 1996). And, the activated Ras interacts with c-Raf, which results in its activation. Although this signaling pathway has not been fully demonstrated in vascular SMC, the ubiquitous distribution of MAP kinases may be extrapolated to SMC, albeit with caution. In accordance, integrin and c-Src activities have been demonstrated in

vascular smooth muscle preparations. The Src-family of nonreceptor tyrosine kinases, which are inhibited by herbimycin A, has been shown to induce ERK 1/2 activation in aortic organ culture albeit in a PKC independent pathway (Birukov et al. 1997). And, Masumoto and colleagues (1997) inhibited the myogenic response with herbimycin A in rat cerebral arteries.

1.4. GAP JUNCTIONS IN THE CARDIOVASCULAR SYSTEM

1.4.1. STRUCTURE AND DISTRIBUTION

A single, functional gap junction unit is comprised of two apposing hexameric hemichannels each of which span the plasma membrane of two adjacent cells. In vascular tissue, these intercellular channels may facilitate communication among smooth muscle cells and endothelial cells, and between the two parenchymal cells types. These hemichannels, or connexons, may contain any combination of a family of transmembrane proteins called connexins. Connexin proteins belong to a multigene family consisting of at least 13 members (Bennett et al. 1994). The molecular weights of these proteins range from 26 to 70 kDa, and subtypes of connexins have been designated according to their molecular weights. Members of this connexin (Cx) family are differentially expressed in tissues, and one cell type may express several connexin subtypes. Vascular smooth muscle cells and endothelial cells have been shown, using immuno-cytochemical light microscopic and immuno-gold studies to express connexin-43 (Cx43 (Christ et al. 1993, 1996).
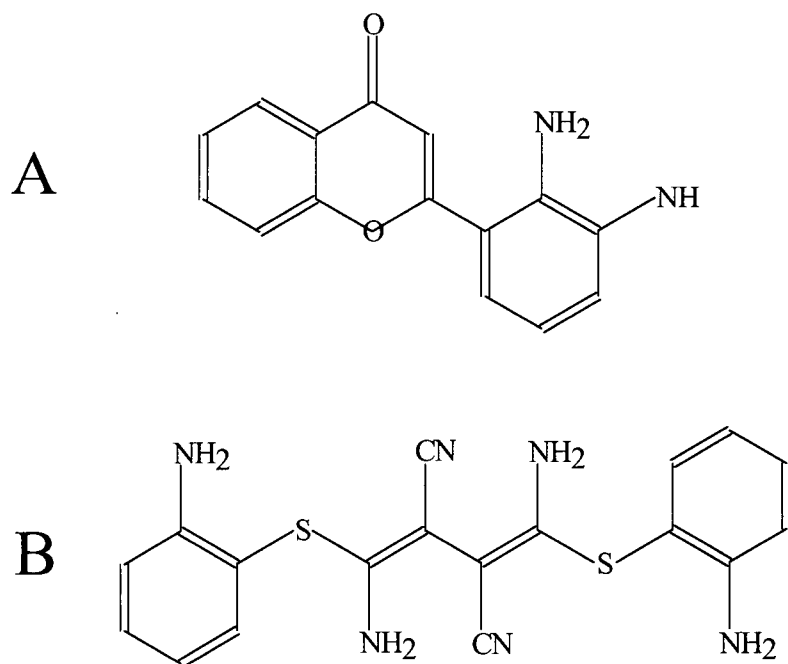
Figure 1.1 Chemical structures of mitogen-activated protein kinase inhibitors. A: PD98059, B: U0126.

Connexin-40 (Cx40) has also been shown to be present in the mammalian arteriolar cell walls and co-aggregate with Cx43 (Little et al. 1995). However, endothelial cells contain an additional subtype, connexin-37 (Cx30). The diversity in Cx expression and specificity with which they are expressed in cells have been postulated to contribute to a given tissue's function.

Each connexin is composed of four α-helical membrane spanning domains (TM1 - TM4), two extracellular loops (E1 and E2) and three cytoplasmic regions (C1, C2, and C3). The extracellular loops and the membrane-spanning domains are highly conserved among species and isoforms (Caterall, 1988). The extracellular loops each contains three highly conserved cysteine residues that form intramolecular disulphide bridges (Dahl et al. 1991) and are essential for the docking process of connexons. Foote et al. (1998) recently proposed a model where the extracellular loops may noncovalently interdigitate with the corresponding loops of an apposing connexin during the docking of the connexons and possibly forming a β-barrel motif within the extracellular space. The extracellular loops are thought to interact via hydrogen bonds, hydrophobic interactions and ionic attraction. Additionally, the second extracellular loop (E2) was demonstrated to be the determinant for Cx compatibility in heterotypic connexon interaction (White et al. 1994).

According to X-ray diffraction studies, a gap junction is a pipe-like structure connecting two neighbouring cells, thus spanning the 20 Å space between the two parallel plasma membranes (Beyer et al. 1995). The channel is 100 – 150 Å long, and the inner pore is approximately 12.5 Å in diameter. Multiple gap junctions

commonly aggregate into islands or plaques of approximately 0.2 μm diameter in corporeal smooth muscle cells (Christ et al. 1993). Christ et al (1993) also estimated the number of gap junctions per plaque to be approximately 100, based on the assumptions that the diameter of a gap junction was 10 nm and the spacing between channels was 5 – 10 nm. There is currently no estimate of the number of plagues per cell.

The distribution of gap junctions in vascular smooth muscle cells is not known. Studies in cardiac cells, however, may provide some insight into their spatial orientation in smooth muscle cells. Under a light microscope, bands of "intercalated discs" traverse the longitudinal axis of cardiac muscle fibers. Within these intercalated discs are clusters of gap junctions that are revealed using the freeze-fracture electron microscopy technique (Makowski et al. 1984). These intercalated discs consist of three structures. The largest part of the intercalated disc is the fascia adherens, which represents the majority of the intercalated disc. It consists of the two plasma membranes of the adjacent cells and is separated by a distance of 200-300 Å. The second structure is the macula adherens that appears as almost a laminate structure. And, the third is the nexus or gap junction that is the region of closet contact between the plasma membranes. This region contains clusters of gap junctions and may comprise 3.3 (Arluk and Rhodin, 1974) to 15% (Hoyt et al. 1989) of the intercalated disc with 11 –12 gap junctions (of a mean profile length of 0.3 μm) occurring per 100 μm along the intercalated disc (Saffitz et al. 1994).

1.4.2. HETEROGENEITY OF GAP JUNCTIONS

The diversity of gap junctions may arise from multiple combinations of connexins due to the highly conserved extracellular loops. The most apparent channel type is the homotypic gap junction in which both connexons are composed of only one type of connexin. Studies of homotypic channels were only possible following the successful cloning of connexins by Paul (1986) and Kumar & Gilula (1986). Typically, biochemical and electrophysiological analyses of specific gap junctions are performed in cells lines devoid of connexin expression such as mouse neuroblastoma (N2a) and human hepatocarcinoma (SKHep1) cells (Kwak & Jongsma, 1999). Adjacent pairs of these cells are either injected with the mRNA for a particular connexin or are transfected with the appropriate cDNA. Alternatively, blocking the endogenous expression of connexins, as for Xenopus oocytes, may produce homotypic channels.

Greater diversity may also occur when one of the two hemichannels consists of a single connexin type; the resulting channel is a heterotypic gap junction. Injecting both cells of a pair with the corresponding connexin cDNA or mRNA typically produces these channels. Studies on heterotypic channels are of physiological relevance since the interaction between two parenchymal cell types may depend on gap junction communication such as at the interface between endothelial and smooth muscle cells (Sandow and Hill, 2000, Fleming, 2000).

The third and possibly the greatest source of heterogeneity may arise when different combinations of connexins combine to form a gap junction. These

heteromeric channels may be formed when cells produce two or more types of connexin, this is the case with endothelial and smooth muscle cells (Christ et al. 1996). Heteromeric channels composed of Cx40 and Cx37 have been found (Bruzzone et al. 1993). Also, heteromeric Cx 43 and Cx 37 gap junctions were present in cells transfected with both connexins (Beyer et al. 1998).

1.4.3. BIOPHYSICAL PROPERTIES

Gap junction conductance is typically measured by isolating cell pairs in culture, and one of the cells is connected to a voltage clamp amplifier. The membrane potential of one cell is held at about −40 mV while the other is clamped to different potentials ranging from −90 to +10 mV (in 200ms steps). Therefore, a transmembrane voltage of ±50 mV can be established. Several electrophysiological studies characterizing gap junction channels formed by the three connexin isoforms (Cx43, Cx40, Cx37) have been performed in expression systems (Reed et al. 1993, Beblo and Veenstra, 1997, Beblo et al. 1995, Bruzzone et al. 1993, Brink et al. 1997, Veenstra et al. 1994). Also, studies in the A7r5 cell line (Moore and Burt, 1995 & 1994, Hirschi et al. 1993, Moore et al. 1991, He and Burt, 1998), cultured arterial (Moore and Burt, 1995), corpus cavernosum (Moreno et al.1993, Brink et al.1996), umbilical cord (Van Rijen et al. 1997), and freshly isolated cerebral arteries (Xing and Marc, 1999) have been done.

The measured currents in both cells (1 and 2) can be described as:

$$I1 = V1/Rm1 + ((V1 - V2) / Rj) \tag{1}$$

and

$$I2 = V2 / Rm2 + ((V2 - V1) / Rj) \tag{2}$$

V1 and V2 are the voltages relative to the holding potential VH. Rm1 and Rm2 are the respective membrane potentials for cells 1 and 2, and Rj is the gap junction resistance. If cell 2 is held at VH, V2 is zero and equations (1) and (2) then becomes:

$$I1 = (V1 / Rm1) + (V1 / Rj) \tag{3}$$

and

$$I2 = -V1 / Rj \tag{4}$$

This double-cell voltage clamp technique can be modified to determine the single

channel conductance (γj) for various connexins. Reducing the coupling of paired cells using a volatile anaesthetic or heptanol allows for the recording of single channel activity. The expression for junctional conductance (gj) is provided below:

$$gj = N \cdot \gamma j \cdot Po \qquad\qquad (5)$$

where the number of functional channels (N), the single channel conductance and the open probability of the channel (Po) are all directly related to the total gap junctional conductance. Studies using this technique have shown that gap junctions homogeneously composed of a single connexin isoform have unique states.

Gap junction channels also possess voltage dependence, as do typical channels. High transmembrane voltage differences (50 – 100 mV), if established for 1 – 2 s decrease gap junction conductance to a steady state conductance (gss) from an instantaneous value (ginst). This voltage dependence can be described using the two-state Boltzmann equation:

$$gss/ginst = \{(gmax - gmin)/(1 + exp[A(Vj - Vo)])\} = gmin \qquad (6)$$

Vo is the voltage at which half of the channels are inactivated, $A = zq / kT$ (z = number of equivalent electron charges, q = voltage sensor, k = Boltzmann constant and T = temperature), gmin is the minimum conductance and gmax is the maximum conductance (which = 1, normalized to the ginst).

Gap junctions allow passage of small molecular weight molecules (<1200 kDa) (Veenstra et al., 1995 and Simpson et al., 1977). Cx40 channels exhibit some selectivity for cations over anions (Beblo et al. 1995); the permeability of Cl⁻ anions was only 29% of K+ cations. There is also strong evidence for that gap junctions allow the diffusion of second messenger molecules such as Ca^{2+}, cAMP, cGMP, and inositol 1,4,5-trisphosphate (Tsien and Weingart, 1976, Sáez et al., 1989, Christ et al. 1992).

Studies on homotypic Cx43, Cx40 and Cx37 gap junction channels have revealed similar and dissimilar properties. For example, while the unitary conductance of each homogenous gap junction is different, they all allow the passage of small molecular weight fluorescent dyes. They all display symmetric voltage dependence as well, but the kinetics and voltage sensitivities are different (Brink, 1996, Veenstra et al. 1995). Heterotypic Cx37 – Cx43 channels, however, do not share symmetrical voltage dependence (Brink, 1998).

1.4.4. BIOCHEMICAL REGULATION

Phosphorylation processes may regulate gap junction conductance via protein

kinases, and up- and down-regulation of cell-to-cell coupling have been reported. Kwak and Jongsma (1996) reported that protein kinase A activation (PKA) with 8-bromo-cAMP had no effect on the coupling of cardiomyocytes or SKHep1 cells transfected with Cx43 (Kwak et al. 1995). In contrast, other studies have shown that PKA activation may enhance gap junction coupling in Cx43-coupled cardiomyocytes (Burt and Spray, 1998, Se Mello, 1988). The basis for these conflicting differences is still questionable and has yet to be resolved.

The activation of protein kinase C (PKC) has been reported to both increase and decrease gap junction coupling. PKC activation causes an increase in g_j in Cx43-coupled cardiac cells but decreases dye coupling in these cells (Kwak and Jongsma, 1996, Spray and Burt, 1990). The discrepancy between electrical and dye coupling has been explained by Kwak et al. (1995) who found that PKC activation decreases the frequency of conductances of the 90 pS state but increases the frequencies of the lower conductance states. Thus, although electrical coupling is increased, the passage of large molecules might be hindered. This explanation may not entirely account for the modulation of gap junction conductance by PKC since other investigators have observed inhibition of electrical coupling following PKC activation (Münster and Weingart, 1992). Protein kinase G (PKG) also affects gap junctional conductances. The activation of PKG with cGMP analogues in cardiomyocytes caused a reduction in the electrical coupling of Cx43 gap junctions and lowered the frequency distribution of the conductance states (Kwak et al. 1995). The roles of tyrosine kinases and MAP kinase in the regulation

of gap junctions have not been studied in the vasculature, but have been shown to

phosphorylate Cx43 channels in other systems (Veenstra et al. 1992, Lau et al.

1996). Additionally, the phosphorylation sites on Cx43 for other protein kinases

have been determined including PKA, PKG, PKC, and calmodulin-dependent

kinase II (Lau et al. 1996).

Small ions such as H^+, Ca^{2+}, Mg^{2+} and Na^+ have also been reported to modulate gap

junctional conductance. Increases in the intracellular concentrations of Na^+, Mg^{2+}

and Ca^{2+} decrease g_j (De Mello, 1975 and 1976, Noma and Tsuboi, 1987, Rüdisüli

and Weingart, 1991, White et al. 1990). A decrease in the intracellular pH can also

reduce the Cx43 gap junction conductance (De Mello, 1976, Maurer and Weingart,

1987) with the histidine residue 95 acting as the pH sensor (Ek at al. 1994).

1.4.5. PHARMACOLOGY OF GAP JUNCTIONS

A diverse variety of structurally distinct compounds have been employed to inhibit

gap junction communication. These inhibitors can be grouped into two general

classes:

lipophilic drugs that incorporate into the plasma membrane and inhibitors that

putatively interact directly with gap junctions. The first class of inhibitors appears to

act by affecting the fluidity of the plasma membrane and disrupt the docking of

connexons. These drugs include fatty acids, volatile anaesthetics (Peracchia, 1991)

and long chain alcohols ($C_7 - C_{12}$). Among these, heptanol (Figure 1.2) is often used

to reduce gap junction coupling so that single channel events can be observed. Johnson et al. (1980) first observed the uncoupling properties of heptanol in crayfish nerves. Heptanol exhibits a steep concentration-effect curve (Hill coefficient = 2.3; Kd = 0.16 mM) using the double-voltage clamp technique (Rüdisüli and Weingart, 1989). And, although its mechanism of action is unknown, heptanol seems to decrease the fluidity of membranous cholesterol domains resulting in a reduced open probability of the gap junction channels (Bastiaanse, et al. 1993, Takens-Kwak et al. 1992). The selectivity of heptanol was reported to be at concentrations less than 2 mM (Kimura et al. 1995), however higher concentrations may affect Na^+ currents in cardiomyocytes (Nelson and Makielski, 1991, Takens-Kwak, 1992). A novel class of inhibitors, glycyrrhetinic acid (GA) (Figure 1.2), has recently been studied, and will be discussed in Section 4.2.

The second class of gap junction inhibitors includes connexin-specific antibodies (Lal et al. 1993, Bastide et al. 1996, Boitano et al. 1998) and peptide fragments analogous to the extracellular loops of connexins (Chaytor et al. 1998, Kwak and Jongsma, 1999). Another class of inhibitors that is putatively selective for gap junctions and gaining acceptance is the aglycone saponin derived from the licorice *Glycyrrhiza spp.*, glycyrrhetinic acid (Davidson and Baumgarten, 1988). Derivatives of glycyrrhetinic acid have been reported to be effective gap junction inhibitors in human fibroblast cell lines (Davidson et al. 1986), liver epithelial cells (Guan et al. 1996), rat lung epithelial cells (Guo et al, 1999) and mesenteric arteries (Yamamoto et al, 1998, Tanaka et al. 1999, Yamamoto et al. 1999). To date, all studies
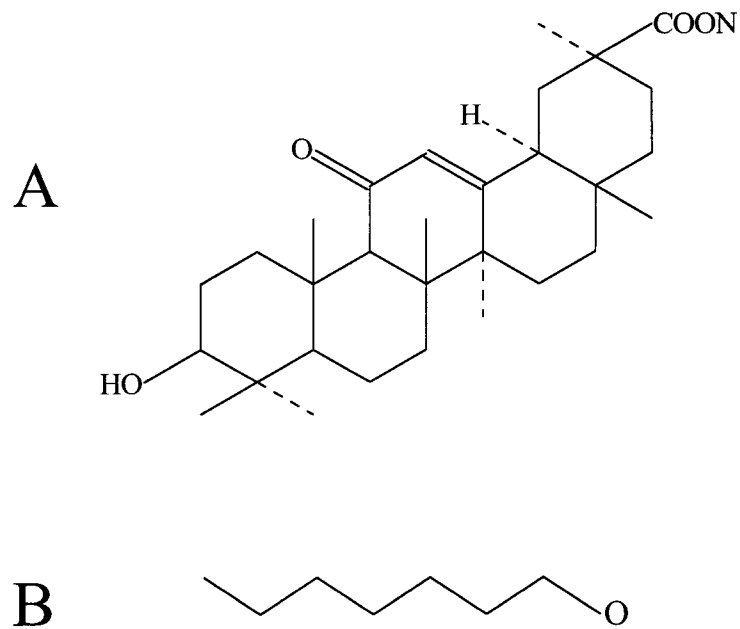
Figure 1.2 Chemical structures of gap junction intercellular communication inhibitors. A: 18α-glycyrrhetinic acid, B: 1-heptanol.

performed on blood vessels with this inhibitor have only examined myoendothelial gap junction communication and its effect on smooth muscle cell-to-cell coupling is not known.

1.5 HYPOTHESES AND OBJECTIVES

HYPOTHESIS 1: Mitogen-activated protein kinases are activated during agonist and myogenic stimulation, and may contribute to the contractility of small arteries.

OBJECTIVE 1: To compare the effects of mitogen-activated protein kinase inhibition using U0126 and PD98059 on myogenic tone, agonist-induced tone and depolarization-induced tone (KPSS).

HYPOTHESIS 2: Gap junction-mediated intercellular communication contributes to the overall activation by agonist or pressure in small arteries.

OBJECTIVE 2: To compare the effects of gap junction intercellular communication inhibition using heptanol and α-glycyrrhetinic acid on myogenic tone, agonist-induced tone and depolarization-induced tone.

2. MATERIALS AND METHODS

2.1. TISSUE ISOLATION, PREPARATION AND DIAMETER MEASUREMENT

Male Sprague-Dawley rats (300-450g) were anaesthetized with sodium pentobarbital (Somnotol, 65mg/kg, i.p.) and administered heparin sulfate (Hepalean, 500 IU/kg) intraperitoneally. The brain was removed and placed into ice-

cold physiological salt solution (PSS) following decapitation. Second-order middle cerebral arteries (150-250μm, intraluminal diameter) were dissected and transferred to a pressure arteriograph vessel chamber (Living Systems, Vt., U.S.A.) containing oxygenated (95% O_2 / 5% CO_2) PSS at room temperature. The superfusing solution was recirculated from an external bath (100 mL) at a flow rate of 15 mL / min. One aspect of the vessel was cannulated with a borosilicate cannula (tip diameter = 70 – 100 μm) and fixed using a single 4-0 nylon thread filament. The intraluminal pressure was minimally increased (3mmHg) using an electronic pressure servo system (Living Systems, Vt., U.S.A.) to gently flush out any remaining blood; the distal end was then similarly cannulated and tied. Under no-flow conditions, intraluminal pressure was elevated to 40 mmHg at which point the bath temperature was raised to 37°C via an external heat exchanger once the absence of leaks was ascertained. The artery segment was equilibrated for 60 min at either 20 or 60 mmHg during which time all vessels reliably developed pressure-induced myogenic tone with significantly reduced luminal diameters.

The vessel chamber was placed onto the stage of an inverted microscope, and vessel diameter was measured using a monochromatic video edge-detection system (Living Systems, Vermont, USA). Intraluminal pressure and diameter were continuously acquired (updated every 17 ms, DATAQ) with the precision of diameter measurements within 1 % and recorded onto a personal computer.

2.2. $[Ca^{2+}]_i$ MEASUREMENT

Briefly, the rat middle cerebral was inverted to expose the endothelial cell side, and tied onto a fixed borosilicate cannula (diameter = 100 – 150 μM). The tissue was loaded in HEPES and incubated with 5 mM membrane-permeant fura 2-acetoxymethyl ester (fura-2AM) and 5 mM pluronic acid to facilitate loading for 1 hour in the dark at room temperature. The bottom of the basilar artery was exposed to alternating 340- and 380- nm (bandwidth 10 nm) ultraviolet light (1/s) that was passed through a 510-nm (bandwidth 40 nm) cut-off filter before acquisition by an ICCD camera (an intensified charge-coupled device; model 4093G, 4810 series, San Diego, CA). The single line trace in each figure is the average of simultaneously measured fluorescence ratio (F_{340}/F_{380}) of individual or groups of smooth muscle cells in the chosen field of the basilar artery preparation. The software program used was Northern Eclipse by Empix Imaging Systems.

Intracellular [Ca^{2+}] measurements were made on the effects of 10 μM U0126 incubated for prior to, and after 30 or 60 min on 40 mM KPSS-induced constrictions.

2.3. EXPERIMENTAL PROTOCOL

To determine the functional role of gap junctions in myogenic tone, heptanol was added to the bath cumulatively (1.0μM – 3.0mM). This range encompassed the previously reported selective concentration required for intercellular uncoupling (Christ, 1995). In two separate series of experiments the agonist vasopressin (10 nM) and a depolarizing 80 mM K^+ PSS (KPSS) were alternately used as preconstricting agents to compare the inhibitory effects of heptanol on different

forms of smooth muscle activation. These experiments were conducted at both low and high pressures (20 and 60 mmHg, respectively); the low pressure was used to minimize the influence of pressure-induced tone. At the end of every experiment, 0 mM Ca^{2+} PSS was substituted to obtain the passive diameter of each vessel. In another set of experiments, 18α-glycyrrhetinic acid (α-GA) was used in place of heptanol. α-GA (1.0 – 50 μM) was added cumulatively to the recirclulating bath in the presence of myogenic tone, agonist-, and depolarization-induced tone. This concentration range was previously reported to be selective for the inhibition of gap junctional communication (Chaytor et al. 1999, Guo et al. 1999). The effects of α-GA inhibition on KPSS- and agonist-induced constriction were examined at 20 mmHg. At the end of these experiments, the superfusate was replaced with 0mM Ca^{2+} PSS to obtain the passive diameters.

To examine the effects of MAPK inhibition on myogenic tone, pressure-diameter relationships in the absence and presence of the inhibitor, U0126 (10 and 40μM), were constructed. Briefly, the transmural pressure of arteries that have been equilibrated at 60 mm Hg was decreased to 10 mm Hg. At this pressure, little or no pressure-induced tone is present in small cerebral arteries, and therefore, passive diameter is generally observed at low pressures. Once the diameter has achieved steady state, pressure was increased to 20 mm Hg and afterwards in 20 mm Hg increments to a maximum of 100 mm Hg. Thus, a control pressure-diameter relationship was determined over a physiological pressure range of 10 to 100 mmHg. This step-wise elevation in pressure is repeated in the presence of 10 or 40

µM of the inhibitor after incubation for 45 min. Passive diameter measurements over this pressure range were then determined by substituting the superfusate with Ca^{2+}-free PSS.

The effects of MAPK inhibition (U0126 or PD98059 at 10 or 40 µM) on agonist-induced and depolarization-induced tone were studied using vasopressin (0.1 µM) and 40 or 60 mM K^+ KPSS, respectively. Tissues that were equilibrated at 20 mm Hg were used in these studies. Control constrictions to either vasopressin or KPSS were initially elicited followed by washout of the buffer and incubation of either 10 or 40 µM of U0126 for 60 min. After the incubation period, either vasopressin or KPSS was reapplied; the inhibitor was present in the replacement depolarizing KPSS. The passive diameter of each vessel was later confirmed by the use of Ca^{2+}-free PSS.

The effects of U0126 on indolactam-potentiated constrictions were also examined. Following vessel equilibration at 60 mm Hg, intraluminal pressure was reduced to 10 mm Hg and allowed to reach steady state. The pressure was then stepped up to 80 mm Hg to induce myogenic tone and indolactam (1.0 µM) was added to the superfusate. Once the new vessel diameter stabilized, the superfusate was replaced to remove the indolactam. This protocol was repeated twice on the same vessel in the presence of 10 and 40 µM U0126.

2.4. MATERIALS AND DATA ANALYSIS

U0126 was given as an investigational tool from Dupont Merck. All other drugs and

materials were purchased from Sigma (MO, USA). PD98059 α-GA and (-)-indolactam V were dissolved in DMSO (stock concentrations = 10 mM) and then aliquoted in separate vials which were frozen until immediately prior to use. (Arg[8])-vasopressin was dissolved in deionized distilled water to a concentration of 100 μM, aliquoted into vials and frozen. U0126, heptanol and α-GA stock solutions were made fresh daily and dissolved in DMSO to a stock concentration of 10 mM, 1.0 M and 10mM, respectively. The composition of PSS was as follows (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.17, KH_2PO_4 1.18, $NaHCO_3$ 24.9, $CaCl_2$ 1.6, EDTA 0.026 glucose 11.1. Equimolar concentrations of KCl and NaCl were substituted to make 40, 60 and 80 mM KPSS. O mM Ca^{2+} PSS was identical to PSS with the exclusion of Ca^{2+} and the addition of 2.0 mM EGTA. All buffers were adjusted to pH 7.4 prior to each experiment and constantly aerated with 95 % O_2 / 5 % CO_2.

Vessel diameters were normalized and expressed either as percentage inhibition or constriction using the equations provided below where dP is the passive diameter and dA is the active diameter.

Percentage Constriction = (dP - dA) × 100 / dP

Percentage Inhibition = 1- [(dP - dA′) / (dP-dA°)] × 100

dA° and dA′ are active diameters in the absence and presence of inhibitor, respectively. Concentration-response relationships were fitted to a sigmoidal dose-

response equation using the Prism2 statistical analysis software package from GraphPad Software Inc. All data are expressed as mean ± S.E.M.. Two-way analysis of variance was used to determine if responses to the inhibitors were different between myogenic-, agonist-, or depolarization-induced tone, and mean values were compared using Student's unpaired t tests. A value of $P < 0.05$ was considered significant.

3. RESULTS

3.1. EFFECTS OF HEPTANOL

KPSS-induced tone at 20 mmHg was inhibited by heptanol in a concentration dependent manner (Figure 3.1); the median inhibitory heptanol concentration (IC_{50}) was 2.0 ± 0.7 mM (n = 4). The threshold for the effect of heptanol relative to control constriction was 2.0mM (p < 0.02), and KPSS-induced tone was inhibited by 77 ± 8% at 3 mM. The concentration-effect curve of heptanol for KPSS at 20 mmHg was therefore very steep (Hill slope = -2.6 ± 0.4) which is in agreement with Rüdisüli and Weingart (1989) who calculated a Hill slope of 2.3 for heptanol in cardiac myocytes. Vasopressin-induced constriction at 20 mmHg was significantly more sensitive to heptanol inhibition than KPSS-induced constriction ($IC_{50} = 400 \pm 300$ μM, n = 7, $p = 0.007$), and a significant inhibition was observed at concentrations as low as 1.0 μM (p < 0.05). Myogenic tone was also inhibited by heptanol ($IC_{50} = 750 \pm 90$ μM, n = 8) and was more sensitive than KPSS-induced tone (p < 0.05). This concentration-

dependence of pressure-induced tone on heptanol was not as shallow as vasopressin (Hill slopes = -1.2 $\pm$ 0.1 and -0.4 $\pm$ 0.2, respectively). Myogenic tone was inhibited at heptanol concentrations of 100 μM (p < 0.008). Myogenic tone was almost completely abolished (97 $\pm$ 1 %) at the highest concentration (3.0 mM), and subsequent washout resulted in an incomplete recovery of tone (36 $\pm$ 8% residual inhibition).

The effects of heptanol inhibition of vasopressin- and KPSS-induced tone at 60 mmHg were also examined (Figure 3.2). At this intraluminal pressure, the arterial segments were myogenically active; vessels exposed to either preconstricting agents (vasopressin or KPSS) were additionally constricted by pressure. Therefore, two distinct forms of vascular smooth muscle cell activation were used to determine the combined sensitivity to heptanol inhibition. Inhibition of KPSS-induced constriction was unaffected by pressure and was nearly identical to the KPSS-inhibition curve at 20 mmHg (IC_{50} = 1.6 $\pm$ 0.5 mM, Hill slope = -1.99 $\pm$ 0.04, n = 7). The combined effects of vasopressin and myogenic tone was less sensitive to heptanol inhibition than either alone. The IC_{50} for vasopressin-induced tone at 60 mmHg was 1.3 $\pm$ 0.4 mM (n = 4) and the corresponding Hill slope was –2.3 $\pm$ 0.4. There was no effect of the solvent at concentrations where KPSS-induced constriction was unaffected. Therefore, it is unlikely that the solvent contributes to heptanol inhibition at the concentrations where selectivity was previously reported ($\approx$300 μM).

3.2. EFFECTS OF 18α-GLYCYRRHETINIC ACID

At high concentrations (> 50 μM), α-GA precipitated in the PSS and caused vasoconstriction. This insolubility prevented the determination of a maximum effect of α-GA. The fitting of the concentration-effect curve was therefore not possible. KPSS-induced tone was unaffected by α-GA (n = 5; Figure 3.4). In contrast, both vasopressin- (n = 4) and pressure-induced (n = 7) vasoconstrictions were inhibited by α-GA and were significantly inhibited at 3 μM (15 ± 4%, p < 0.04 and 5 ± 2 %, p < 0.03; respectively) relative to KPSS. Although not significantly different from each other, agonist-induced vasoconstriction was relatively more sensitive to α-GA inhibition than myogenic tone.

3.3. EFFECTS OF U0126 AND PR98059

The acute effects of U0126 resulted in a concentration dependent inhibition of myogenic tone at 60 mmHg with a median inhibitory concentration (IC_{50}) of 21 ± 6 μM (Figure 3.5; n = 4). Vasopressin- and KPSS-induced constrictions were also inhibited in a concentration dependent manner (IC_{50} = 20 ± 30, n = 5 and 91 ± 5, n = 5, respectively). Both agonist-induced constriction and myogenic tone were significantly more sensitive to U0126 inhibition in comparison to depolarization-induced constriction ($P < 0.0005$).

In experiments where U0126 was incubated for 60 min, the lower concentration of U0126 (10 μM) only significantly affected the pressure-diameter relationship at 60 mm Hg (Figure 3.6) where the percentage constriction was reduced to 44% of

control (18 ± 3%) in the presence of 10µM of the inhibitor (8 ± 3%). At the higher

concentration of the inhibitor (40µM), myogenic tone was almost completely ablated

throughout the physiological pressure range. In comparison to the lower

concentration of U0126, percentage constriction at 60 mm Hg was reduced to 7 %

of control (25 ± 3 %, control; 1.3 ± 0.6 %, 40µM U0126) in the presence of 40 µM

U0126.

Vasopressin-induced constriction was inhibited by 10µM of U0126 after 60 min of

preincubation. The control constriction was decreased from 58 ± 4% to 6 ± 5% in

the presence of 10µM of the inhibitor. Depolarization-induced constriction (control =

50 ± 4%) was not significantly inhibited by U0126; however, the level of constriction

elicited by KPSS was reduced with increasing concentrations of U0126 (45 ± 4%,

10µM; 28 ± 9%, 40µM U0126). Therefore, U0126 appears to inhibit VGCC at

concentrations greater than 10 µM. PKC-potentiated tone (control = 68 ± 6%) was

not significantly affected by 10µM of U0126 (63 ± 4%). However, indolactam-

induced tone was almost completely inhibited by 40µM of the inhibitor (6 ± 4%).

Preliminary measurements on Ca2+ effects suggest a time dependence for the

inhibitory effects of U0126. 40 mM KPSS was not affected by 10 µM U0126 after a

30 min preincubation (Figure 3.8). In comparison, after 60 min, U0126 (10 µM

almost completely inhibited KPSS-induced constrictions (Figure 3.9).

Figure 3.1. Heptanol inhibition of induced tone in the pressurized rat middle cerebral artery. Inhibitory log heptanol concentration response relationships of myogenic tone (60 mmHg, n = 8, squares), vasopressin-induced (20 mmHg, n = 7, triangles) and KPSS-induced (20 mmHg, n = 4, diamonds) tones. Values are mean ± S.E.M., and *P < 0.05 compared with KPSS.
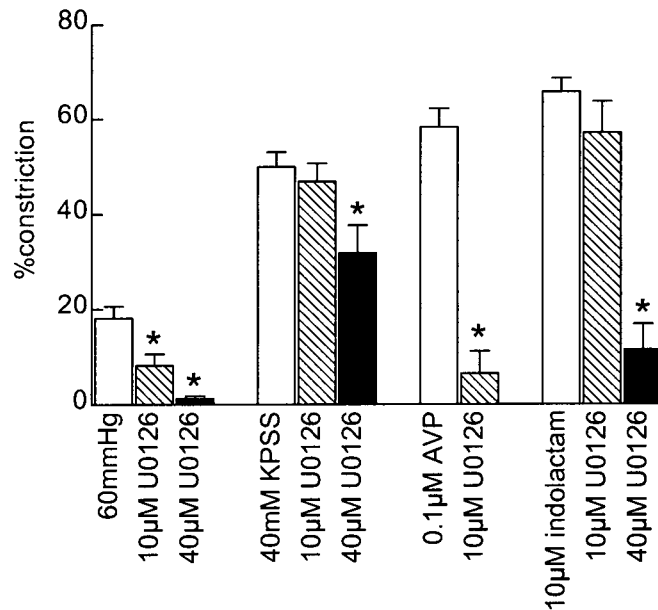
Figure 3.2. Heptanol inhibition of vasopressin and 80 mM K$^+$ physiological salt solution (KPSS) in combination with myogenic tone. Inhibitory log heptanol concentration response relationships of vasopressin-induced (60 mmHg, n = 4, triangles) and KPSS-induced (60 mmHg, n = 7, diamonds) tones. Values are mean ± S.E.M.

20min

1.4

340/380

0.4

40mM KPSS

10 μM U0126
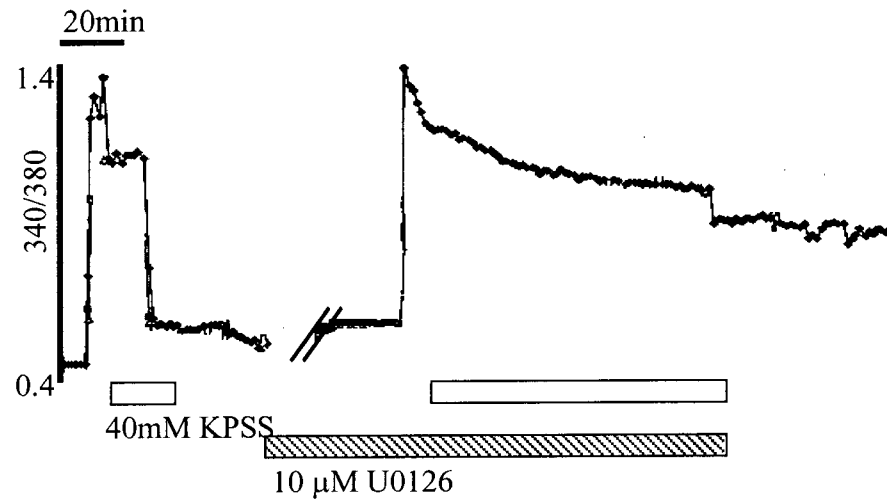
Figure 3.3. Relative diameters of pressurized rat middle cerebral arteries after preconstriction. Pressure-induced tone was significantly less than tone induced by 80mM K^+ physiological salt solution and 0.1 μM vasopressin (at 20 mmHg). Values are mean ± S.E.M., and *P < 0.05.
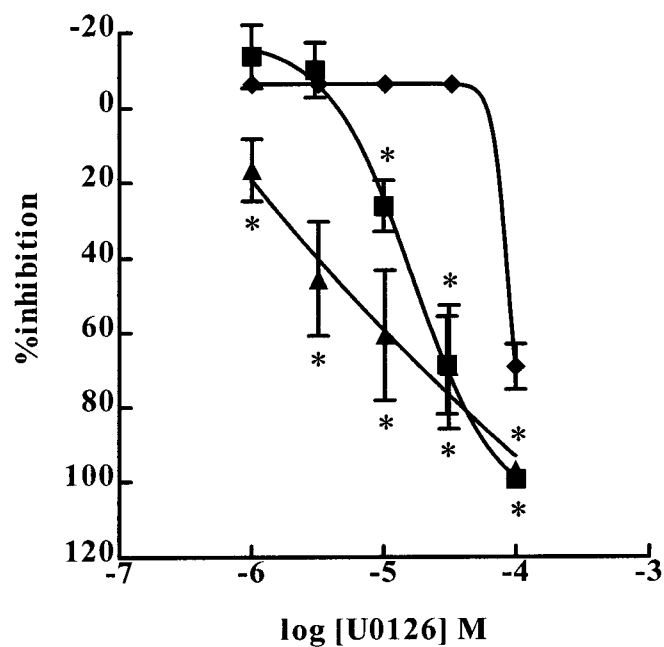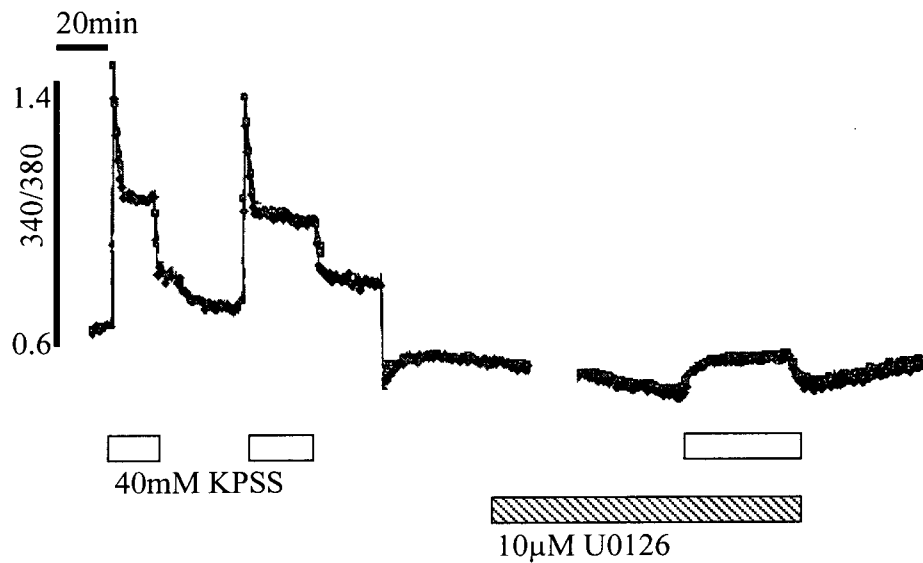
Figure 3.4. α-Glycyrrhetinic acid (α-GA) inhibition of induced tone in the pressurized rat middle cerebral artery. Inhibitory log α-GA concentration response relationships of myogenic tone (60 mmHg, n = 7, squares), vasopressin-induced (20 mmHg, n = 4, triangles) and KPSS-induced (20 mmHg, n = 5, diamonds) tones. Values are mean ± S.E.M., and *P < 0.05 compared with KPSS.
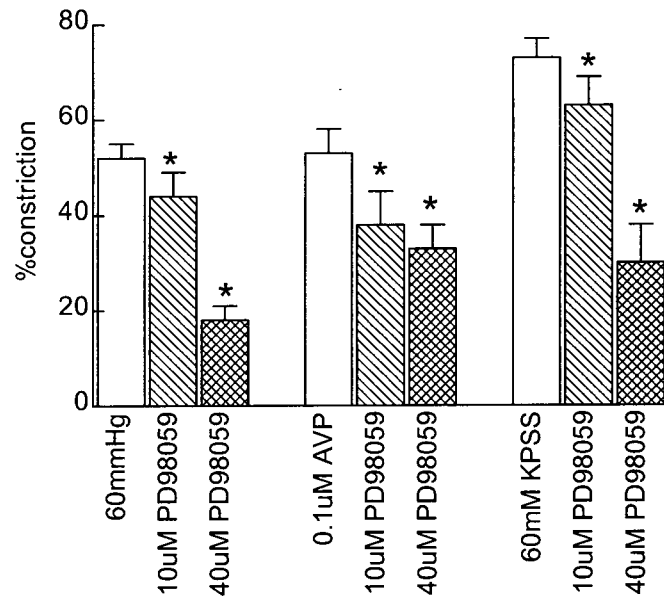
Figure 3.5. U0126 inhibition of induced tone in the pressurized rat middle cerebral artery after 10 min. Inhibitory log U0126 concentration response relationships of myogenic tone (60 mmHg, n = 4, squares), vasopressin-induced (20 mmHg, n = 5, triangles) and 40 mM KPSS-induced (20 mmHg, n = 5, diamonds). Values are mean ± S.E.M., and *P < 0.05 compared with KPSS.
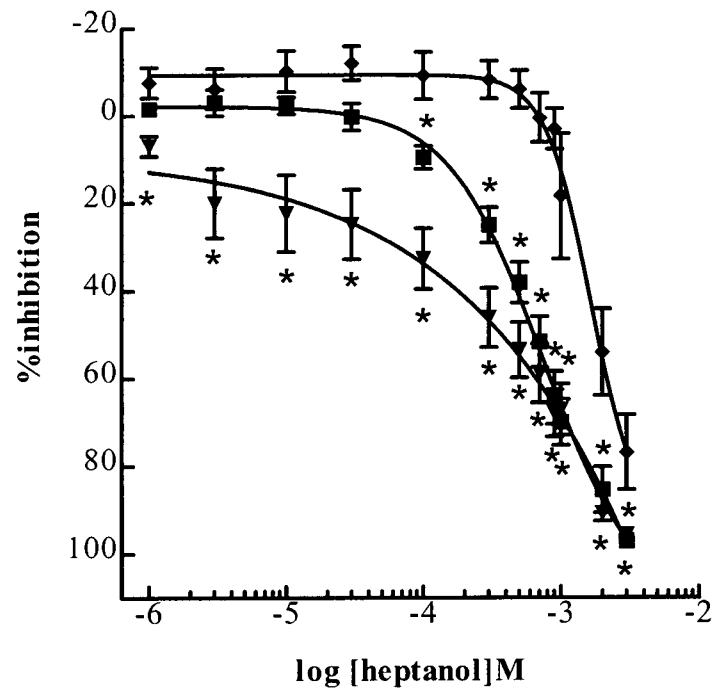
Figure 3.6. U0126 inhibitory effects on induced tone in the pressurized rat middle cerebral artery after 60 min. U0126 (10 μM) significantly inhibited myogenic, vasopressin-induced and indolactam-potentiated tone after 60 min of preincubation. U0126 was nonselective at 40 μM. Values are mean $\pm$ S.E.M., and *P < 0.05 compared with control response.
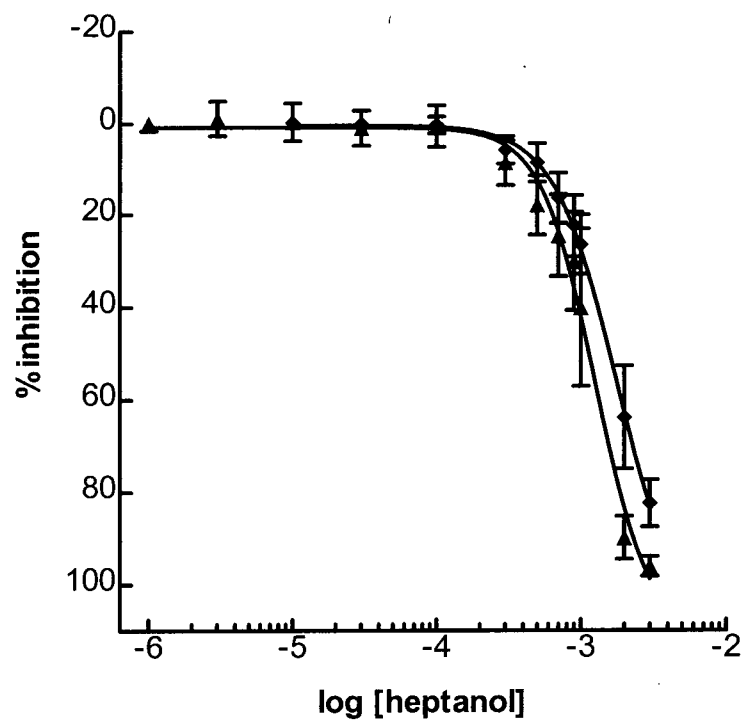
Figure 3.7. PD98059 inhibitory effects on induced tone in the pressurized rat middle

cerebral artery after 60 min. PD98059 significantly inhibited myogenic, vasopressin-

and 60 mM KPSS-induced tone after 60 min preincubation. Values are mean ±

S.E.M., and *P < 0.05 compared with control response.
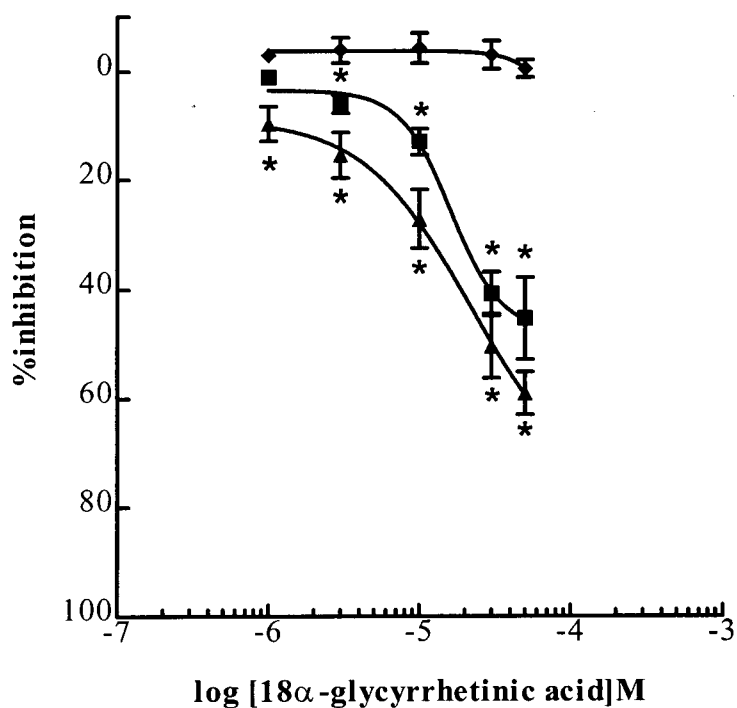
Figure 3.8. Representative trace of $[Ca^{2+}]_i$ in response to 40mM K^+ physiological salt solution before and after 30 min preincubation with 10 μM U0126. Depolarization-induced Ca^{2+} influx in the rat middle cerebral artery was unaffected after 30 min exposure to 10 μM U0126.
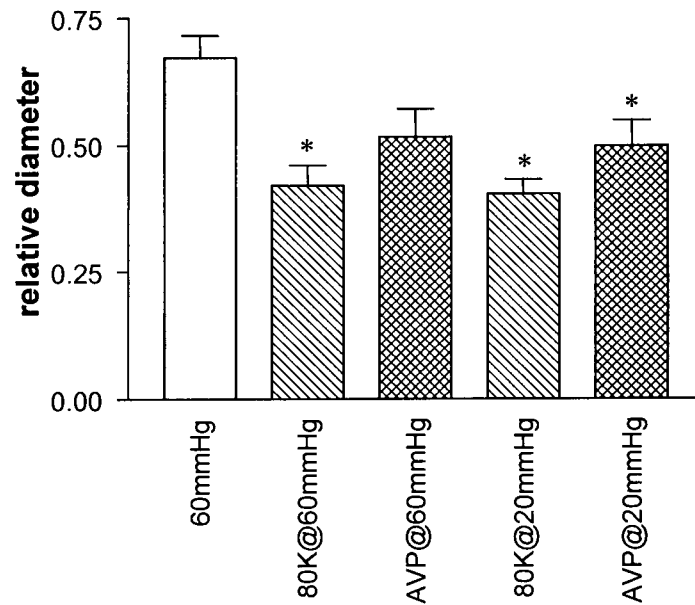
Figure 3.9. Representative trace of $[Ca^{2+}]_i$ in response to 40mM K^+ physiological salt solution before and after 60 min preincubation with 10 μM U0126. Depolarization-induced Ca^{2+} influx in the rat middle cerebral artery was inhibited after 60 min exposure to 10 μM U0126 indicating a nonspecific inhibition of voltage-gated Ca^{2+} channels.

PD98059 inhibited myogenic tone by 15% at 10 μM (from 52 $\pm$ 3% to 44 $\pm$ 5%; n = 6), and by 64% at 40 μM (Figure 3.7; from 52 $\pm$ 3% to 18 $\pm$ 3%, n = 6). Another series of experiments was conducted to verify the specificity of PD98059 as an inhibitor of MAP kinase. Tone was induced by vasopressin (0.1 μM) and 60 mM K^+ PSS which served as positive and negative controls, respectively, for the specificity of PD98059 as an inhibitor of MEK. Vasopressin-induced constriction was inhibited by 30% (from 53 $\pm$ 5% to 3 $\pm$ 7%; n = 6) and 38% (from 53 $\pm$ 5% to 33 $\pm$ 5%; n = 6) after the addition of 10 and 40 μM, respectively. PD98059 also inhibited the constriction caused by KPSS; 10 μM inhibited constriction by 13 % (from 73 $\pm$ 4 % to 63 $\pm$ 6 %; n = 6), and 40 μM PD98059 further reduced the constriction by 60 % (from 73 $\pm$ 4 % to 30 $\pm$ 8 %; n = 6).

4. DISCUSSION

4.1. MAP KINASE INHIBITION

One of the primary findings of this study was that MEK inhibition with U0126 at 10 μM, caused discriminate inhibition of four different forms of induced tone in pressurized resistance arteries. However, a 4-fold increase in this concentration resulted in nonspecific, and in most cases complete inhibition of constriction. PD98059 was relatively nonselective as this compound inhibited all forms of vasoconstrictions evaluated. Therefore, the value of PD98059 as a pharmacological tool for determining the functional effects of MAP kinase is limited.

4.1.1. MAP KINASES AND Ca^{2+} SENSITIVITY?

Although the primary mechanisms for pressure and agonist-induced vasoconstriction appear to share the common pathway of elevated cytosolic $[Ca^{2+}]$ and increased Ca^{2+} sensitivity (Hill et al. 1996, VanBavel et al. 1998, Gokina et al. 1998), the intermediary between these two events has not been established. Activation of the ERK 1/2 isoforms has been postulated to mediate the increased Ca^{2+} sensitivity of the myofilaments by directly phosphorylating and thereby disinhibiting the thin-filament associated regulatory protein, caldesmon (Adam et al., 1995, 1992; Franklin et al., 1997). The thin filament proteins, caldesmon and calponin are known to inhibit actomyosin ATPase activity *in vitro* (Ngai and Walsh, 1984, Sobue et al. 1985, Szpacenko et al. 1985, Marston et al. 1988, Abe et al. 1990, Winder and Walsh, 1990). The addition of a C-terminal 20 kDa caldesmon fragment to skinned smooth muscle fibers inhibits force and shifts the dependence of force on phosphorylation towards higher phosphorylation levels (Pfitzer et al. 1993). In stretched carotid arteries, caldesmon phosphorylation levels paralleled the rise in ERK kinase activity (Franklin et al.1997). Taken together, the phosphorylation of thin filament regulatory proteins by ERK kinase may relieve this inhibition and shift the $[Ca^{2+}]$-force relationship to the left. By preventing ERK 1/2 phosphorylation and activation with MEK 1/2 inhibitors, the functional effect of ERK inhibition was indirectly determined. The finding that MEK inhibition moderately reduced the level of myogenic constriction and, to a greater extent vasopressin-

induced constriction corroborates with the above conjecture. Pressure, as a stimulus for vasoconstriction, may only incur a small shift in contractile protein Ca^{2+} sensitivity compared to agonist-induced constriction. However, the scope of the present study did not include measurements of cytosolic $[Ca^{2+}]$ to constriction relationships, and therefore the effect of ERK activation on Ca^{2+} sensitivity during the myogenic response remains speculative.

4.1.2. PKC AND MAP KINASE INHIBITION

The lack of effect of U0126 on indolactam-potentiated constriction at a concentration that selectively inhibited myogenic- and agonist-induced tone suggests that PKC may not be directly involved in the MAP kinase cascade. PKC plays a role in the pressure- or agonist-induced enhancement of Ca^{2+} sensitivity by either activating the downstream ERK 1/2 (Franklin et al., 1997) or altering the contractile proteins through an ERK-independent pathway. In support of this hypothesis, Loufrani et al. (1999) concluded that stretch in the rabbit facial vein induced two distinct pathways, one leading to myogenic tone (via a non-calcium-dependent PKC activation) and one leading to ERK 1/2 activation involving a tyrosine kinase. At least two possible PKC-mediated events that lead to an increase in smooth muscle Ca^{2+} sensitivity have been proposed. Calponin, another thin filament regulatory protein, is phosphorylated during PKC-induced contraction in porcine coronary arteries (Mino et al. 1995) and may therefore result in a disinhibition of actomyosin ATPase similar to caldesmon. Another MAP kinase

independent pathway for PKC-induced constriction may be through the inhibition of myosin phosphatase either directly or by phosphorylation of an endogenous inhibitor (Somylo et al. 1989, Itoh et al. 1993). Recent evidence demonstrates a PKC-dependent phosphorylation of CPI-17, a potent inhibitor (when phosphorylated) of the catalytic subunit of myosin light chain phosphatase (PP-1C; Eto et al. 1995, Kitazawa et al. 1999, Senba et al. 1999). Furthermore, PKC may modulate voltage-gated Ca^{2+} and K^+ channels in addition to affecting the level of sensitivity of the contractile proteins (Galizzi et al. 1987, Hill et al. 1996, Cole et al. 1996, Kirton and Loutzenhiser, 1998) therefore compounding the complexity of intracellular signaling in the resistance arteries. Thus, PKC may be involved in the autoregulation of resistance arteries but possibly through pathways divergent from the MAP kinase cascade.

4.1.3. SELECTIVITY OF INHIBITORS

The selectivity of MEK kinase inhibitors was contingent upon its inability to inhibit depolarization-induced tone that presumably elicits smooth muscle contraction without affecting the Ca^{2+} sensitivity. This study examined the effects of MEK inhibition in a more physiological context than in previous studies measuring isometric tension with wire myographs. At 40µM, U0126 nonselectively inhibited pressure, agonist, PKC, and depolarization-induced tone. All four results may be related, since the development of depolarization-induced tone depends on the opening of VGCC; therefore, at high concentrations, U0126 may inhibit L-type Ca^{2+}

channels. In comparison, the other MEK 1/2 inhibitor, PD98059 is nonspecific and may also have inhibitory effects on VGCC (Lagaud et al. 1999) at concentrations below that which inhibits ERK activity *in vitro* (Loufrani et al 1999). Although these compounds are structurally dissimilar, they noncompetitvely bind to MEK, with respect to MEK substrates, ATP and ERK and in a mutually exclusive manner indicating a common or shared bind site(s) (Favata et al 1998). In addition, U0126 reportedly has a 100-fold selectivity over PD98059 for MEK and does not affect the activities of other kinases. The selectivity of U0126 and not PD98059 underscores the advantage of this compound as a pharmacological tool, however more studies are required to determine the specificity of this inhibitor. However, the possibility that U0126 may inhibit receptor-operated Ca^{2+} channels (ROCC) cannot be excluded since agonists mediate vasoconstriction via the activation of VGCC, PKC and ROCC. The near complete inhibition of vasopressin-induced constriction by U0126 (at 10 μM), and the relative lack of effect on KPSS- and indolactam-induced vasoconstrictions suggests that either ROCCs are inhibited by the compound or that ROCCs do not contribute to vasopressin-induced constrictions. Moreover, the differences observed between the acute (10 min) effects of U0126 and following incubation of 60 min may be the result of nonspecific inhibition or incomplete diffusion into the cytosol after 10 min. Preliminary $[Ca^{2+}]_i$ measurements using fura-2 of the effects of U0126 (10 μM) on Ca^{2+} influx indicated a time-dependent inhibition of KPSS-induced $[Ca^{2+}]_i$ increase after 60 min (Figure 5).

4.1.4. SUMMARY

In summary, MEK 1/2 inhibition with U0126 caused the inhibition of myogenic- and vasopressin-induced tone without affect high K^+ constrictions. PKC-potentiated constrictions were also unaffected at the same concentration. However, PD98059 and higher concentrations of U0126 (40 μM) nondiscriminately inhibited all constrictions possibly through the inhibition of voltage-gated Ca^{2+} channels.

4.2. GAP JUNCTION INHIBITION

This study demonstrates, for the first time, the sensitivity of myogenic and agonist-induced tone to inhibitors of gap junction communication in resistance arteries. The data suggest a functional role for intercellular coupling in resistance arteries and confirms previous immunochemical and molecular biological studies demonstrating the presence of gap junctions in microvessels (Little et al. 1995, Xing and Simard, 1999). Furthermore, these results indicate that inhibition with heptanol or α-GA can selectively disrupt the global reactivity of small, resistance-sized arteries to receptor activation and/or G protein-mediated events.

4.2.1. SIMILARITY BETWEEN AGONIST AND PRESSURE STIMULI

Myogenic tone is a property that allows small arteries to autoregulate blood flow to the capillary beds. This property has been demonstrated in a variety of animal and human vascular beds including the cerebral, coronary, and renal systems (for reviews, Johnson, 1989, Faraci et al. 1989, Roman and Harder, 1993, Bevan and

Laher, 1991 Muller et al. 1996, Schubert and Mulvany, 1999, Davis and Hill, 1999). Pressure-induced stretch causes an endothelium-independent depolarization of smooth muscle cells and an opening of L-type voltage-gated Ca^{2+} channels (Knot and Nelson, 1995, 1998,). Also, phospholipase C is activated via G-protein(s) and produces the second messenger, inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from the sarcoplasmic reticulum to also contribute to the rise in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Concomitantly, diacylglycerol (DAG), another product of the PLC-mediated reaction, can activate PKC, which is involved in Ca^{2+} sensitization of the contractile filaments (Bevan and Laher, 1991). These and other divergent signaling mechanisms of vascular smooth muscle cells must be conducted to adjacent cells in order for the whole vessel to constrict in syncytium. The finding that inhibitors of gap junction intercellular communication discriminately inhibits both myogenic (at 60 mmHg) and vasopressin-induced (at 20 mmHg) tones at lower concentrations than that of KPSS-induced constriction reflects the importance of intracellular signaling in the mechanotransduction of pharmacological and physical stimuli in resistance-sized arteries and that cell-to-cell propagation of these signals may be required. Furthermore, these analogous results from structurally dissimilar inhibitors strongly suggest that a distinct and perhaps common mechanism modulates both myogenic and agonist-induced tone.

The similar effects of heptanol and α-GA on agonist and pressure-induced vasoconstriction may be the result of a nonspecific inhibition at the level of the agonist receptor or wall tension sensor. Intracellular signaling for agonists and

possibly pressure originates at the plasma membrane and are coupled to G-proteins. Vasopressin receptors (V_1) are linked to $G_{\alpha q}$-proteins that are coupled to PLC (Thibonnier et al. 1993, Holtzman and Ausiello, 1994); in addition, the both agonist and integrin receptors may be coupled to monomeric GTP-binding proteins such as Ras (Sadoshima and Isumo, 1993). Following receptor activation, the signal diversifies into numerous second messenger cascades culminating in an elevation of $[Ca^{2+}]_i$ and smooth muscle contraction. This shared origin for both forms of VSM cell activation is a possible site of inhibition since both inhibitors are lipophilic and reportedly act at the plasma membrane (Takens-Kwak et al. 1992, Bastianne et al. 1993, Christ 1995, Goldberg et al. 1996, Guan et al. 1995, Yamamoto et al. 1998, Yamamoto et al. 1999). Along the lines of this hypothesis, the lack of inhibition of α-GA and reduced efficacy of heptanol on KPSS-induced constriction suggests that receptor-mediated vasoconstriction may not be directly coupled to VGCC. Thus, these results indicate that the myogenic response and agonist activation of SMC probably follow similar pathways. Nonetheless, the possibility that gap junctions may have a more critical role in these responses cannot be excluded.

4.2.2. INHIBITION OF GAP JUNCTIONS?

An alternative mechanism may account for the partial inhibition of myogenic tone and vasopressin-induced constriction with heptanol and α-GA; gap junction communication may be critical for the recruitment and/or potentiation of contraction

of pressure or agonist insensitive SMCs. Therefore, the uncoupling of gap junctions may limit the transmission of electrical or biochemical signals from stimulus-sensitive cells to those that are insensitive and dependent on signal propagation for activation. This hypothesis is conditional upon an unequal distribution of receptors and/or insensitive cells among the population of SMCs. In accordance, Graham and Keatinge (1972) found differences in the sensitivity of adrenoceptor stimulation across the sheep carotid artery wall indicating the presence of relatively insensitive SMCs. Functional intercellular channels have been shown to be permeable to cations and anions including Ca^{2+} (Christ et al. 1992), therefore presumably facilitating the propagation of Ca^{2+} waves to adjacent cells (Sneyd et al. 1994). Additionally, small molecular weight solutes (300 to 1200 Da) such as dyes, second messengers and metabolites can pass through the pore (Simpson et al. 1977, Veenstra et al. 1995). Heptanol has been postulated to inhibit gap junction communication at the level of the plasma membrane, either through a hydrophilic binding by the polar hydroxyl group to the connexon or by decreasing the fluidity of cholesterol-rich membrane domains and facilitating a physical dissociation of coupled hemichannels (Bastianne et al. 1993, Takens-Kwak et al. 1992). Inhibition of gap junctions either by a direct uncoupling or downregulation of junctional gating properties is probable since these channels remain mostly in the open state, and therefore, the contribution of the low-conductance state(s) is minimal (Brink, 1996, Brink et al. 1996, Burt and Spray, 1988, Christ, 1995). Glycyrrhetinic acid similarly acts at the level of the plasma membrane. However, it binds to surface proteins

(Negishi et al. 1991) and may therefore interact directly with the gap junction channel (Davidson and Baumgarten, 1988). Disruption of intercellular communication with GA derivatives was also paralleled by dephosphorylation of Cx43 (Guan et al. 1995) and disruption of gap junction plaques (Goldberg et al. 1996) indicating a selective inhibitory interaction with gap junctions. Electrical coupling between mesenteric smooth muscle cells and across the myoendothelial interface was also inhibited by a GA derivative (Yamamoto et al. 1998, Yamamoto et al. 1999).

This mechanism may account for heptanol's reduced efficacy at 60 mmHg. Incomplete uncoupling may limit the transmission of electrical or chemical signals from sensitive cells to those that are insensitive and dependent on signal propagation for activation. The combined effects of both agonist and pressure on both populations of cells would then increase the effective number of activated cells. It would therefore be possible to estimate the fraction of responsive cells from the level of residual constriction provided the sub-maximal concentration of heptanol is selective for gap junctions. The proportion of vasopressin- or pressure-insensitive SMCs in the rat middle cerebral artery may alternately be approximated with the effects of α-GA (Figure), assuming that it is specific for the inhibition of gap junction communication. Several recent studies report the sensitivity of gap junctions to inhibition by GA. In cultured rat alveolar epithelial cells, α-GA completely inhibited dye coupling at 5 μM (Guo et al. 1999). Spontaneous rhythmical contractions and depolarizations in the irideal arteriole of the rat were

abolished at 20 μM of the inhibitor (Hill et al. 1999). Moreover, a derivative of α-GA, 18β-glycyrrhetinic acid blocked electrical communication across the myoendothelial space at 40 μM in guinea pig mesenteric arterioles (Yamamoto et al. 1998, Yamamoto et al. 1999). Therefore, at a concentration that has been reported to completely inhibit gap junction communication (30 μM), the proportion of SMCs that are dependent on cell-to-cell communication for agonist and pressure-induced vasoconstriction may be predicted to be approximately 50 and 41%, respectively. However, the actual distribution of vasopressin receptors in this tissue has not yet been determined, and the wall tension sensor remains to be identified.

The decreased efficacy of heptanol for inhibiting vasopressin at 60 mmHg (Figure 2) as indicated by an increase in IC_{50} may also be due another mechanism. Gap junction-permeable second messengers such as Ca^{2+}, IP_3, and 20-HETE that are produced because of stretch may contribute to greater contractile activation of cells. Also, as intraluminal pressure increases, membrane potential depolarizes (Kirton and Loutzenhiser, 1998); this effect would enhance the influx of Ca^{2+} and lead to greater contractility. Agonist-induced PKC activation may additionally potentiate vasoconstriction caused by pressure; agonists such as angiotensin II and endothelin-1 that activate PKC, potentiate pressure-induced constriction in resistance arteries at low, sub-constrictor concentrations (Knot and Nelson, 1998). Therefore, the synergistic effects of pressure and agonist stimulations may increase the contractility of SMCs thereby compensating for the reduced cell coupling caused by heptanol.

4.2.3. SPECIFICITY OF INHIBITORS

Rat cerebral arteries of the size used in these experiments are relatively thin walled. Under these conditions, the rapid diffusion of the extravascular solution into the vascular wall would cause all excitable cells to depolarize independent of each other; this effect would otherwise be time-dependent in larger arteries (Christ et al. 1999). The inhibitory effect of heptanol on KPSS-induced constriction likely indicates its nonjunctional effects. Vasoconstriction to KPSS relies upon the activation of L-type voltage-gated Ca^{2+} channels on smooth muscle cells, and the inhibition of KPSS-induced constriction would therefore suggest an interference of Ca^{2+} influx by altering L-type Ca^{2+} channel properties and / or the contractile filaments. Recent studies by Garcias-Dorada and colleagues (1997) in isolated rat cardiomyocytes confirm the relative selectivity of heptanol at concentrations ranging from 30 to 300 μM; the nonjunctional effects of heptanol were reported at concentrations greater than 1 mM. Additionally, Watts et al. (1994) demonstrated that at, 10 μM, heptanol inhibited cell-to-cell dye transfer in cultured rat mesenteric smooth muscle cells by 75%. Thus, at concentrations where others have reported selectivity at inhibiting gap junctions, heptanol is relatively selective due to its ability to discriminate between different forms of vasoconstriction (Figures 1 and 3). However, complete inhibition of gap junctions may not occur at concentrations where VGCC are unaffected since Yamamoto and others (1998) reported the abolishment of myoendothelial electrical communication in guinea pig mesenteric

arterioles at concentrations of 1 mM and greater. The lack of inhibition of KPSS-induced constriction by α-GA suggests that this novel inhibitor does not affect Ca^{2+} channels and is more selective than heptanol in this regard (Figure 3).

4.2.4. SUMMARY

In summary, this study demonstrates the sensitivity of myogenic and agonist-induced tone to inhibitors of gap junctions, suggesting that gap junction communication in resistance arteries contribute to the maintenance of these forms of vasoconstriction. Both inhibitors of intercellular communication attenuated constrictions at concentrations that did not significantly affect KPSS-induced tone although these compounds exhibited narrow windows of efficacy or selectivity. And, in spite of the lack of data on the specificity of these inhibitors, the similarities of their effects on agonist- and pressure-induced responses provide evidence for a shared receptor-mediated signaling pathway. These findings delimits the usefulness of heptanol as a pharmacological tool for investigating the role of gap junctions and supports the selectively of a relatively new class of gap junction inhibitor.

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