UNIVERSITY OF ALBERTA

ROLE OF PROTEIN KINASE C IN CEREBROVASCULAR SPASM

BY

GRANT RAYMOND WICKMAN 

A thesis submitted to the Faculty of Graduate Studies and Research in the partial fulfillment of the requirements for the degree of Master of Science

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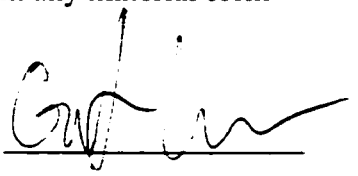

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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled *Role of protein kinase C in cerebrovascular spasm*, submitted by Grant Raymond Wickman in partial fulfillment of the requirements for the degree of Master of Science

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All nature is but art, unknown to thee;

All chance, direction, which thou canst not see;

All discord, harmony not understood;

All partial evil, universal good;

And spite of pride, in erring reason's spite;

One truth is clear, Whatever is, is right.

Alexander Pope

To mom

Without you this work would not have been possible. I thank you for all your sacrifices, love, and encouragement. You will never be alone or forgotten.

I will always love you.

Grant

Abstract

The pathogenesis of cerebral vasospasm is unclear. Evidence suggests that oxyhemoglobin (OxyHb) and endothelin-1 (ET-1) may cause vasospasm and protein kinase C (PKC) may play a role in the vasoconstrictive action of these compounds. The role of PKC in vasoconstriction is a major focus of this thesis. The ability of tamoxifen and the aminoglycoside antibiotics to reverse vasoconstriction has also been studied.

Both OxyHb and ET evoked translocation of PKC isoforms and enhanced the activity of membrane bound PKC. Sustained vasoconstriction of cerebral arteries was inhibited by specific PKC inhibitors. Tamoxifen and the aminoglycosides attenuated OxyHb and ET-1 induced vasoconstriction, at least in part, via PKC inhibition.

These studies indicate that PKC plays a key role in the vasoconstriction induced by OxyHb and ET-1. The ability of tamoxifen and the aminoglycoside antibiotics to reverse vasoconstriction induced by these agents suggests that they may be useful in the treatment of cerebral vasospasm.

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ANOVA	analysis of variance
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium ion concentration
CaD	caldesmon
CaM	calmodulin
CaP	calponin
CSF	cerebrospinal fluid
CVSMC	cerebrovascular smooth muscle cell
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
ECE	endothelin converting enzyme
EDTA	ethylenediaminettraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ERK	extracellular signal regulated kinase
ET	endothelin
ET_A	endothelin A receptor
ET_B	endothelin B receptor
cGMP	cyclic guanosine monophosphate
GPCR	G-protein coupled receptor
IC_{50}	Concentration of antagonist which causes 50 % inhibition of agonist response

IP_3	inositol (1,4,5) trisphosphate
kDa	kiloDalton
LC_{20}	20 kDa myosin light chain
MAPK	mitogen activated protein kinase
MetHb	methemoglobin
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
L-NAME	Nω-nitro-L-arginine methyl ester
NO	nitric oxide
eNOS	endothelium-nitric oxide synthase
iNOS	inducible-nitric oxide synthase
nNOS	neuronal-nitric oxide synthase
NP-40	nonidet P-40
OxyHb	oxyhemoglobin
PA	phosphatidic acid
PC	phosphatidylcholine
PS	phosphatidylserine
PIP_2	phosphatidyl inositol (4,5) bisphosphate
PKC	protein kinase C
PKM	protein kinase M
PLC	phospholipase C
PLD	phospholipase D

PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
SR	sarcoplasmic reticulum
SDS-PAGE	sodium-dodecyl-sulphate poly acrylamide gel electrophoresis
SAH	subarachnoid hemorrhage
s.e.m.	standard error of the mean
TAM	tamoxifen

CHAPTER 1:

1.0 GENERAL INTRODUCTION: CEREBRAL VASOSPASM

1.1 INTRODUCTION

Subarachnoid hemorrhage (SAH) is a serious pathological disorder most commonly caused by intracranial aneurysmal rupture. Approximately 2% of the population harbor intracranial aneurysms, which is a weakening and ballooning out of the vessel wall (Sekhar *et al.*, 1981). The rupture of such a vessel presents a serious life-threatening condition, and a substantial proportion of patients die within the first 12 h after aneurysmal rupture (Kassell *et al.*, 1982). One of the most serious complications following SAH is cerebral vasospasm, or delayed onset cerebral arterial narrowing (Weir, 1995). This condition was first described by Ecker and Riemenschneider in 1951 and remains the leading cause of morbidity in patients with SAH (Ecker and Riemenshneider, 1951). Currently there is no treatment which can effectively prevent or reverse the cerebral vasoconstriction (Macdonald, 1995). Vasospasm can be divided into two groups, angiographic and clinical. It is estimated that approximately 60-70% of SAH patients display angiographic spasm; however, approximately 30% of those patients will experience the clinical manifestations of the spasm (Kassel *et al.*, 1985). One possible explanation of this apparent discrepancy between the rates of angiographic and clinical vasospasm is that clinical vasospasm is not only dependent on arterial narrowing of the major cerebral arteries, as detected by angiography, but also on the collateral circulation and blood flow (Kassel *et al.*, 1985 ; Kassel *et al.*, 1985). Furthermore, it is believed that, for symptomatic vasospasm to develop, cerebral arterial narrowing must be greater than 50 % thereby increasing associated cerebral ischemia (Kassel *et al.*, 1985). The onset of cerebral vasospasm after SAH frequently occurs between the third and fourth day, peaks on the seventh to tenth day, and is resolved by day fourteen (Weir *et al.*, 1978 ; Torner *et*

al., 1990). Clinical manifestations of vasospasm are signs of cerebral ischemia and include neurological deficit, persistent or increasingly severe headache, and decreased level of conscienceness and coma, which may be followed by focal neurological signs such as speech difficulties and hemiparesis (Macdonald. 1995 ; Rousseaux *et al.*, 1980).

While vasospasm has been widely studied, the processes leading to sustained vessel contraction are not well understood. There is a growing consensus that the release of spasmogens from lysing erythrocytes within the clot is the most important factor in the etiology of vasospasm. This is consistent with the time course of erythrocyte lysis which coincides with the development of vasospasm (Pickard *et al.*, 1992). Furthermore, additional evidence shows that the location and amount of clotted blood present in the subarachnoid space correlates with the location and severity of the spasm (Adams, 1992).

Recently there have been significant advances made in the treatment of cerebral vasospasm following SAH. Current treatment consists of aneurysm clipping and clot removal, transluminal balloon angioplasty, and hypertensive hypervolemic therapy involving vasoconstrictors, volume expanders, and calcium channel antagonists. Nonetheless, the therapies listed above are only partially effective in preventing or reversing vasospasm and protecting the brain from its ischemic consequences (Kassel, 1993). The lack of efficacy of the current therapies may be attributed to a lack of understanding of the molecular processes underlying vasospasm, and as a consequence, 15% of clinical vasospasm patients either die or have permanent disability (Kassell *et al.*, 1990). Therefore, there is a clear need for a better understanding of the pathogenesis of cerebral vasospasm and an effective therapy to prevent or reverse the arterial narrowing associated with SAH.

1.2 PATHOGENESIS OF VASOSPASM

The pathogenesis of vasospasm stems from a narrowing of the lumen of cerebral arteries following SAH, which in turn leads to cerebral ischemia and infarction that is ultimately responsible for the morbidity and mortality associated with the disorder. Two major hypotheses have been advanced to explain the luminal narrowing; the first theory suggests that structural changes in the vessel wall result in thickening and therefore narrowing of the lumen (Findlay *et al.*, 1989), the second proposes that vasospasm is a prolonged and temporarily irreversible smooth muscle contraction that leads to structural changes of the vessel wall. In 1964 Crompton *et al.*, reported that vessels from patients with angiographically confirmed vasospasm who had died more than 3 weeks following SAH had structural pathomorphological changes. However, the vessels in this report were analyzed at a time when vasospasm had resolved and therefore the significance of these observations remained unclear. Since that report many groups have studied the structural changes in the cerebral vasculature post SAH and found early reductions in luminal size followed by smooth muscle cell migration and proliferation, intimal thickening, and eventually tissue necrosis (Fein *et al.*, 1974 ; Mayberg *et al.*, 1990). However, in 1985 Pickard and co-workers failed to detect any structural changes in cerebral vessels shown to have angiographic spasm in a canine model of experimental vasospasm. Thus, it would appear that the structural changes follow sustained vasospasm, suggesting that the vascular remodeling is a result rather than a cause of vasospasm (Hughes *et al.*,1978). Therefore, it has become accepted that vasospasm is a state of smooth muscle contraction, while the changes in the vessels walls reflect a response of the arteries to a prolonged contractile stimulus.

As previously mentioned there is an excellent correlation between the location and extent of the bleeding and the location and severity of cerebral vasospasm. Furthermore, the time course of erythrocyte lysis parallels the time course of vasospasm development. Thus, the nature of cerebral vasospasm suggests that agents released from the lysing erythrocytes within the subarachnoid blood clot are responsible for the disorder and the resulting sequelae. While many spasmogens have been implicated in the pathogenesis of vasospasm, attempts to pin point a single causative agent have been unsuccessful. Currently there is growing evidence which suggests that oxyhemoglobin (OxyHb) and the vasoactive peptide endothelin-1 (ET-1) are the primary spasmogens involved in the development of cerebral vasospasm following SAH.

1.2.1 Major mediators of vasospasm

1.2.1.1 Oxyhemoglobin

Multiple lines of evidence suggest that oxyhemoglobin (OxyHb) is the principal etiologic agent of cerebral vasospasm. Oxyhemoglobin is known to have a profound contractile effect on isolated cerebral arteries (Cook *et al.*, 1979 ; Tanishima, 1990 ; Wellum *et al.*, 1982). Moreover, OxyHb is present in the cerebral spinal fluid (CSF) of patients following SAH in concentrations sufficient to mediate prolonged vasoconstriction (Pluta *et al.*, 1998). OxyHb also stimulates prolonged increases in intracellular free calcium lasting for several days and stimulates morphological changes in cultured cells consistent with changes in spastic vessels (Takanashi *et al.*, 1992 ; Vollrath *et al.*, 1995). Furthermore, in animal models, OxyHb and the supernatant fluid

from lysed erythrocytes produce sustained vasospasm when injected *in vivo*, while reduced methemoglobin and the hemoglobin breakdown product, bilirubin, remain ineffective (Macdonald *et al.*, 1991). Therefore, it is widely accepted that OxyHb released via erythrocyte lysis from the subarachnoid clot is a key mediator of cerebral vasospasm.

While it is evident that OxyHb is involved in the pathogenesis of vasospasm the mechanisms by which it initiates prolonged contraction are unclear. Many potential mechanisms have been proposed and at present three hypothesis stand out. First, OxyHb-induced generation of free radicals and subsequent generation of lipid peroxides may be ultimately responsible for cerebral vasospasm following SAH (Steele *et al.*, 1990). Second, scavenging of nitric oxide by OxyHb may attenuate endothelium-mediated vasorelaxation, which, may in turn, trigger vasoconstriction (Hongo *et al.*, 1988). Third, OxyHb-stimulated production and secretion of the potent vasoactive peptide, endothelin-1 (ET-1), may induce a powerful vasoconstriction of the cerebral arteries (Kasuya *et al.*, 1993).

1.2.1.1.1 FREE RADICALS

The observation that MetHb, which contains ferric iron, is inactive as a spasmogen while OxyHb, which contains iron in the ferrous form, consistently produces vasoconstriction suggests that the oxidation state of iron is a critical determinant of spasmogenic activity. Consequently, it has been proposed that ferrous iron in OxyHb may participate in an iron-catalyzed production of free radicals, a so-called Haber-Weiss reaction (Halliwell and Gutteridge, 1986). The autoxidation of OxyHb to MetHb is

associated with the release of a superoxide radical which reacts with water to form hydrogen peroxide (fig 1). Hydrogen peroxide then interacts with the ferrous iron present in hemoglobin to produce the highly toxic hydroxyl radical (Wever *et al.*, 1973). The hydroxyl radical, in turn, initiates and propagates lipid peroxidation of plasma membranes which is known to mediate a variety of cellular responses (Gutteridge and Halliwell, 1990). The formation of lipid peroxides has been shown to impair membrane function, change membrane permeability, induce membrane blebs, and eventually lead to cell death (Steele *et al.*, 1990). The observation that MetHb does not induce lipid peroxidation or smooth muscle contraction is consistent with the proposal that OxyHb-induced contractile effects are dependent upon free radical formation. Ultimately the effects of lipid peroxides may be responsible for initiation and maintenance of intracellular events that cause contraction of vascular smooth muscle associated with vasospasm.

There is some evidence to support the concept that OxyHb vasoconstrictor activity is mediated through free radical production and subsequent formation of lipid peroxides. Intracisternal injections of a xanthine oxidase/ xanthine mixture, which is commonly used as a source of oxygen radicals, has been shown to initiate sustained cerebrovascular vasoconstriction (Kamiyama *et al.*, 1981) In addition, OxyHb promotes the formation of lipid peroxides, which are recognized to possess contractile activity (Koide *et al.*, 1982). Injection of lipid peroxides has been shown to produce vasoconstriction and structural damage of cerebral arteries in animal models (Sasaki *et al.*, 1981). Furthermore, the concentration of lipid peroxides in the cerebrospinal fluid of patients is increased during vasospasm which suggests a direct link between OxyHb free

radical production and subsequent lipid peroxidation (Sasaki *et al.*, 1979 and 1980). This evidence is consistent with the observation that OxyHb is a potent spasmogen while MetHb is largely devoid of contractile activity.

Further evidence implicating free radicals in the pathogenesis of cerebral vasospasm is derived from the observation that compounds with antioxidant activity may attenuate vasospasm following SAH. It has been hypothesized that the enzymes superoxide dismutase (SOD) and catalase, which are responsible for the conversion of superoxide anion to hydrogen peroxide which is then degraded by catalase, may exert a protective effect and ameliorate vasospasm (Kamii *et al.*, 1999). Although some research groups have reported that the SOD and catalase were effective in inhibiting vasospasm (Kamaiyama *et al.*, 1981) other studies have failed to show protective effects (Macdonald *et al.*, 1992 ; Wellum *et al.*, 1982). It has been suggested that the lack of efficacy of SOD and catalase in these studies may be due to the inability of these large enzymes to reach the site of free radical production where they may exert positive effects. In support of this proposal human recombinant SOD, which has a high plasma membrane penetrating capacity, was shown to be effective at inhibiting experimental vasospasm when injected into the subarachnoid cisterns (Shishido *et al.*, 1993). 21-aminosteroid compounds, such as tirilizad mesylate, are scavengers of oxygen free radicals and effective inhibitors of lipid peroxidation and therefore may be of some benefit in the treatment of vasospasm (Braughler *et al.*, 1987). It has been proposed that the steroid nucleus stabilizes the membrane which may shield membrane proteins and inhibit the propagation of lipid peroxidation by restricting free radical movement within the membrane (Braughler *et al.*, 1988 and 1989 ; Hall *et al.*, 1994.). Another potentially beneficial effect of the 21

aminosteroids is the chelation of ferrous iron. It has been demonstrated that these compounds are potent inhibitors of iron-catalyzed lipid peroxidation, an effect which may be attributed to iron chelation (Braughler et al., 1987). The compound tirilizad has been shown to ameliorate vasospasm both in experimental models and clinical trials (Steinke et al., 1989 ; Vollmer et al., 1989 ; Kassel et al., 1996). This evidence lends additional support to the hypothesis that free radicals and lipid peroxidation are involved in the pathogenesis of cerebral vasospasm. Nonetheless, free radical production and lipid peroxidation is not the sole mechanism proposed for OxyHb mediated vasospasm.

1.2.1.2 NITRIC OXIDE (NO)

There is some evidence to suggest that OxyHb may interfere with vasorelaxant nitric oxide (NO) signaling pathways which may lead to vascular contraction. Since the classic work of Furchgott and Zawadzki (1980) it has been recognized that the endothelial cells which line the lumen of all arteries release mediators of smooth muscle relaxation. Endothelium mediated vasodilation occurs via release of endothelium derived relaxing factor (EDRF), identified as nitric oxide (NO) (Ignarro *et al.*, 1987). NO is formed during the conversion of L-arginine to L-citrulline by the catalytic action of three well characterized enzymes termed NO synthases (Palmer *et al.*, 1988). The first type, endothelial NOS (eNOS), is constitutive, calmodulin-dependent NOS, and is abundantly expressed in vascular endothelial cells (Moncada *et al.*, 1991). The second type, is neuronal NOS (nNOS) and is primarily expressed in neuronal tissue, and therefore it is thought not to be involved in vasospasm. The third form of NOS is inducible (iNOS) and its expression is stimulated following exposure to immunologic or inflammatory stimuli.

Expression of the inducible form of NOS may be anticipated in injured vascular tissue after SAH due to tissue damage and subsequent release of proinflammatory mediators. However, some results suggest that the iNOS expression in cerebrovascular smooth muscle cells remains unchanged and therefore this enzyme is not a factor effecting vasospasm (Suzuki et al, 1989). NO, when produced in endothelial cells rapidly diffuses to smooth muscle cells where it produces vascular relaxation by binding to the heme moiety in the active site of soluble guanylate cyclase in smooth muscle. Activation of this enzyme results in the generation of cyclic GMP which has a relaxant effect in smooth muscle (Schmidt *et al.*, 1993). Alternatively, NO might also mediate a direct effect on smooth muscle by binding to and activating K^+ channels thereby maintaining a hyperpolarized state (Butler *et al.*, 1995 ; Hunley *et al.*, 1995).

Vascular tone is essentially a balance between opposing contractile and relaxant factors, and it has been established that inhibition of the action of NO can enhance the efficacy of contractile agonists. OxyHb, the principle agent implicated in the etiology of vasospasm, possesses the ability to bind and inactivate NO (Martin et al., 1985). Thus it has been proposed that the ability of OxyHb to bind NO may play a role in the pathogenesis of cerebral vasospasm following SAH.

If OxyHb, released following SAH, is involved in the inactivation of NO then NO donor compounds , such as glyceryl trinitrate, might be expected to reverse the scavenging effects of OxyHb and prove to be beneficial in the treatment of vasospasm. However, the evidence is controversial and in some cases NO donors appear to be ineffective inducers of relaxation (Onoue at al., 1995 ; Kim et al., 1992 ; Yamamoto et al., 1997 ; Sobey et al., 1996). It has been proposed that the lack of efficacy of NO

donors may be attributed to impairment of NO-mediated responses and some studies show decreased basal levels of cGMP associated with cerebral arteries after SAH (Onoue et al., 1995.). Nonetheless, these observations suggest that OxyHb-induced contraction is not dependent on nitric oxide scavenging. However, OxyHb-mediated scavenging of NO would be expected to amplify the contractile response to OxyHb due to loss of vasorelaxant stimulus (Cook and Vollrath, 1995). Therefore, while NO scavenging by OxyHb is likely to effect vasospasm its impact remains unclear.

1.2.1.3 ENDOTHELIN-1

The vascular endothelium is not only responsible for initiating smooth muscle relaxation, but also releases numerous constrictor substances. In 1988 Yanagisawa et al. isolated and sequenced a vasoactive peptide from endothelial cell cultures capable of producing prolonged vasoconstriction lasting more then 60 min. This substance has been termed endothelin and is the most potent vasoconstrictor known to date. Subsequent studies have shown that endothelin is a member of a family of three 21 amino acid vasoactive peptides which include endothelins 1, 2, and 3 (Inoue et al., 1990). Each isoform contains two disulfide bridges linking cysteine residues. These bonds appear to be critical for the actions of the peptides as their removal leads to substantial loss of activity (Randall et al., 1989). The endothelins are synthesized as preproendothelin which is cleaved by an endopeptidase to yield the 38 amino acid big endothelin, a less active peptide than the mature endothelin. The final processing step leading to fully active ET is an intracellular cleavage catalyzed by the ET converting enzyme (ECE) (Turner and Murphy, 1996). Although discovered as products of vascular endothelial

cells the endothelins have now been shown to be produced in many cell types, including vascular smooth muscle cells (Hahn et al,. 1990). Once produced ET is secreted abluminally and is capable of acting in a paracrine or autocrine manner (Schiffrin and Touyz, 1998).

ETs have been found to bind with high affinity to two identified heptahelical transmembrane receptor subtypes, ET_A and ET_B. Binding of ETs to their receptors is thought to reach a steady state within 10-20 min and dissociation of the ligand is exceptionally slow. The high affinity and slow dissociation of ET binding to its receptors may confer the long-lasting vasoconstrictor effect of the peptide. The ET receptors display different affinities for the various ET peptides; ET_A receptors bind ET-1 and ET-2 with greater affinity than ET-3, while ET_B receptor appears to be non-selective and binds all ET peptides with equal affinity (Arai et al., 1990 ; Sakurai et al., 1992). Distribution of these receptor subtypes is tissue and cell specific; ET_A receptors are expressed abundantly on vascular smooth muscle cells while endothelial cells primarily express ET_B (Schifferin and Touyz, 1998). Regardless of the subtype, all ET receptors are Gq-protein coupled and activation stimulates diverse signaling pathways leading to multiple cellular effects, including vascular smooth muscle contraction (fig 3). Activation of ET receptors stimulates the phosphatidylinositol-4,5-bisphosphate signaling pathway by activation of membrane bound phospholipase C_β (PLC_β). Activation of PLC_β results in the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to two second messengers, water soluble IP_3 and membrane bound diacylglycerol (DAG), which are ultimately responsible for many of the effects of ET (fig 3). Endothelin induces large increases of intracellular free calcium ($[Ca^{2+}]_i$) which are typically biphasic comprising a rapid initial

transient phase and a sustained plateau phase (Simpson and Ashley, 1989 ; Touyz et al., 1994). The rapid phase is primarily generated by IP_3-induced mobilization of intracellular calcium and, to a lesser extent, by Ca^{2+}-induced Ca^{2+} release. The plateau phase, which appears to play a major role in the sustained ET-1-induced vasoconstriction, is dependent on Ca^{2+} influx through both receptor and voltage operated Ca^{2+} channels as well as on PKC activity. ET-induced DAG production initiates the activation of protein kinase C (PKC), a family of serine/threonine kinases whose activity has been implicated in sustained vascular contraction. In addition to its powerful vasoconstrictive action, ET is recognized to be a powerful mitogen in vascular smooth muscle. The mitogenic effect of ET likely involves the activation of tyrosine kinases; in particular the src family of nonreceptor tyrosine kinases are thought to be involved in ET stimulated activation of mitogen activated protein kinase (MAPK), and action which leads to alteration of gene expression and to cell growth (fig 3)(Aramori and Nakanishi, 1992 ; Simonson *et al.*, 1992 ; Yanagisawa and Masaki, 1989).

The observation that endothelin produces a powerful prolonged contraction of arteries has attracted a special interest in relation to vasospasm. It has been suggested that ET may act as a key factor in the development of cerebral vasospasm following SAH (fig 3), and many studies have been performed to investigate this possibility. ET is a potent powerful contractile agonist of isolated cerebral arteries (Ide *et al.*, 1989 ; Saito *et al.*, 1989). In addition, ET levels in the CSF of patients following SAH are elevated, suggesting a potential involvement of this peptide in the development of vasospasm. However, these studies remain controversial (Cosentino and Katusic, 1994 ; Hamann *et al.*, 1993 ; Roux *et al.*, 1995 ; Seifert *et al.*, 1995). Furthermore, intracisternal injections

of ET induce a prolonged contraction of canine cerebral arteries that resembles sustained vasospasm (Asano *et al.*, 1989 ; Kobayashi *et al.*, 1990). In animal models of vasospasm, ET levels in the basilar artery are significantly increased by day 2 (Yamaura *et al.*, 1992). In addition, the vasospasm was also moderately reversed by the topical application of monoclonal antibodies against ET-1 (Yamaura *et al.*, 1992). It has also been shown that ET receptor antagonists may prevent or reverse vasospasm. Two day exposure to BQ-123, a selective ET_A receptor antagonist, has been shown to completely prevent cerebral artery contraction following induction of vasospasm in animal models (Itoh *et al.*, 1994). Numerous other studies have reported beneficial effects of ET_A receptor antagonists in cerebral vasospasm (Itoh *et al.*, 1993 and 1994 ; Nieri *et al.*, 1993). Animal studies have demonstrated that intracisternal exposure of cerebral arteries to phosphoramidon, an inhibitor of the endothelin converting enzyme (ECE), prior to induction of vasospasm significantly decreases the severity of the spasm (Matsumara *et al.*, 1990 ; Matsumara *et al.* 1991, ; Shinyama *et al.*, 1991). In another set of experiments, Onoda *et al* (1996) blocked ET synthesis using highly specific prepro-ET-1 mRNA antisense oligonucleotides and significantly inhibited vascular contraction following 20 min exposure to hemolysate. This evidence suggests that ET may play an central role in the development of vasospasm (Matsumara *et al.*, 1991). The observations that OxyHb is a potent stimulator of endothelin production in both endothelial cells and smooth muscle cells provides an important link between these two spasmogens in the pathogenesis of vasospasm (Kasuya *et al.*, 1993). The effect of OxyHb on ET production has been shown to be mimicked by PMA, a PKC activator, and reduced by staurosporine, a PKC inhibitor. Therefore, it has been suggested that OxyHb induced ET production is

regulated by a protein kinase C dependent mechanism (Kasuya *et al.*, 1993 ; Ohlstein and Storer, 1992).

1.3 MECHANSIMS OF VASCULAR SMOOTH MUSCLE CONTRACTION

Stimulation of vascular smooth muscle contraction leads to a classic biphasic response, consisting of a rapid induction of contraction followed by sustained maintenance of developed tone. The initiation of contraction is triggered by an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$). Intracellular free Ca^{2+} initiates a signaling cascade by binding to calmodulin (CaM), a ubiquitous and multifunctional calcium binding protein. This interaction induces a conformational change in CaM such that it can then bind and activate intracellular targets (Klee, 1980). In the context of smooth muscle contraction the most important target for activated CaM is myosin light chain kinase (MLCK). This protein serine/threonine kinase displays a high specificity for serine 19 in the 20-kDa light chain (LC_{20}) of the myosin hexamer. Currently the only known substrate of MLCK is the myosin light chain (Horowitz *et al.*, 1996). In the resting state MLCK is folded in such a way that the pseudosubstrate domain within the carboxyl terminus is bound to the enzyme's active site maintaining MLCK in an inactive conformation (Pearson *et al.*, 1988). Within the pseudosubstrate domain lies an overlapping CaM-binding site; when activated CaM binds to MLCK a conformational change is induced whereby the active site may no longer bind the pseudosubstrate allowing full activation of the enzyme (Kemp *et al.*, 1987). The phosphorylation on LC_{20} by MLCK triggers activation of the myosin associated Mg^{2+} dependent ATPase activity,

thus allowing crossbridge cycling between actin and myosin filaments and leading to force development. Relaxation of smooth muscle contraction is catalyzed by the dephosphorylation of LC_{20} by myosin light chain phosphatase (MLCP) (Shirazi *et al.*, 1994). Thus, it can be seen that smooth muscle contraction is a direct result of the myosin light chain phosphorylation. Since myosin's phosphorylation state is governed by the action of both MLCK and MLCP it follows that the relative activities of these enzymes are ultimately responsible for smooth muscle contraction.

There are at least two distinct mechanisms leading to the increase in intracellular calcium which triggers contraction. The principal mechanism of intracellular calcium elevation is a rapid release of calcium from intracellular stores. This release mechanism is an integral part of agonist stimulated pharmacomechanical coupling leading to contractile responses. Many agonists such as endothelin, $PGF_{2\alpha}$, and noradrenaline are capable of initiating such responses by binding to G-protein coupled receptors and triggering phospholipase C (PLC) activation. As previously mentioned, this process initiates the hydrolysis of inositol phospholipids and the subsequent generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3, once generated, initiates a rapid release of Ca^{2+} from the intracellular stores via the specific IP_3 receptor on the sarcoplasmic reticulum (SR) to trigger contraction (Berridge, 1993). DAG causes the activation of PKC isoforms which, as will be discussed later, also play a role in vascular contraction (Nishizuka, 1992 ; Berridge and Irvine, 1989). The second mechanism which contributes to the rise in $[Ca^{2+}]_i$ associated with smooth muscle contraction is influx from the extracellular space. Extracellular calcium influx in vascular smooth muscle occurs via membrane depolarization and subsequent activation of voltage operated dihydropyridine

sensitive L-type Ca^{2+} channels leading to smooth muscle contraction, a process called electromechanical coupling (Fasolato *et al.*, 1994).

Although the importance of Ca^{2+}/CaM-dependent myosin phosphorylation as a critical step in smooth muscle contraction is unquestionable, it is now recognized that during prolonged physiological stimulation there is a substantial component of smooth muscle contraction that occurs at near basal $[Ca^{2+}]_i$. Experiments have demonstrated that the force/Ca^{2+} ratio is variable during agonist induced contraction (Himpens *et al.*, 1990), indicating that smooth muscle is capable of modulating its responsiveness to the $[Ca^{2+}]_i$. This observation suggests that Ca^{2+}/CaM dependent myosin phosphorylation is a less critical component of prolonged tone maintenance, suggesting alternative mediators of contraction.

The sustained phase of smooth muscle contraction is characterized by DAG accumulation, which has been identified as the endogenous activator of PKC isoforms (Nishizuka, 1992). Like vascular contraction, DAG production has also been identified as biphasic. Initial transient production of DAG results from the hydrolysis of inositol phospholipids, a process which is then followed by sustained production of DAG from the hydrolysis of phosphatidylcholine (PC), the major membrane phospholipid. Hydrolysis of PC is mediated by phospholipase D (PLD) which produces phosphatidic acid, which is then converted by the action of a specific phosphohydrolase to DAG (Lassegue *et al.*, 1993). This process is not associated with an increase in $[Ca^{2+}]_i$, thus allowing a prolonged activation of PKC at low $[Ca^{2+}]_i$ (Rassmussen *et al.*, 1987). The observation that PKC may undergo prolonged activation during sustained contraction implies a potential role for this enzyme in smooth muscle contraction. A large body of

evidence supports PKC mediated vasoconstriction. Phorbol esters have long been recognized for their ability to potently stimulate many PKC isozymes by binding and mimicking DAG. Exposure of vascular smooth muscle to phorbol esters produces a slowly developing powerful prolonged contraction. This evidence suggests that activation of PKC is sufficient to initiate smooth muscle contraction. The involvement of PKC in sustained smooth muscle contraction has also been demonstrated on the basis of the finding that application of the constitutively active catalytic fragment of this enzyme, known as protein kinase M (PKM), to permeabilized aortic cells induced a prolonged contraction (Collins *et al.*, 1992). Further evidence is provided by the observation that both phorbol ester-induced contraction and PKM-induced contraction could be reversed with a synthetic inhibitory peptide, a selective inhibitor of PKC (Collins *et al.*, 1992).

The protein kinase C (PKC) enzymes consist of a single polypeptide chain with an amino-terminal regulatory site and a carboxy-terminal kinase domain (Nishizuka, 1995 ; Newton, 1995). Isoforms of PKC are categorized into three groups, classical, novel and atypical, based on their requirements for activation. The classical isoforms were the first to be identified and consist of the α, βI-BII, and γ isoforms. Activation of these isoforms is regulated by DAG, phosphatidylserine, and free Ca^{2+} (table 1). The novel class of PKCs is represented by the δ, ε, η, θ, isoforms which are also activated by DAG and phosphatidylserine (PS) yet lack a calcium binding site and therefore remain insensitive to Ca^{2+} (table 1). Atypical PKCs include the ζ, and λ isoenzymes and, although their regulation has not been fully elucidated, it is recognized that their activity is stimulated by PS (table 1). In the inactive unstimulated state PKC enzymes are locked in an inactive conformation by the binding of the autoinhibitory pseudosubstrate domain

to the catalytic site. Depending on the type of PKC the regulatory domains contain binding sites for PS, DAG, and Ca^{2+} in addition to the pseudosubstrate sequence. Activation of PKC requires specific binding of DAG and PS which, in turn, triggers calcium binding. This complex series of events allows for the high affinity membrane interactions that result in enzyme activation. It is important to note that the two major lipid cofactors for PKC activation, DAG and PS, are located in the membrane due to their hydrophobicity. Therefore active PKC is membrane bound by virtue of the lipid cofactors required for its activation. This membrane translocation that PKCs undergo is considered a hallmark of their activation.

There have been numerous targets proposed for PKC-mediated contraction of vascular smooth muscle. One of the most promising candidates is the actin binding protein calponin (CaP). When bound to actin, CaP decreases the capacity of actin to activate myosin ATPase (Miki *et al.*, 1992), and prevents contraction (fig 2). It has been suggested that phosphorylation of CaP by PKC removes CaPs ability to bind actin, thereby relieving CaP-induced inhibition of contraction. When applied exogenously to phenylephrine-stimulated skinned smooth muscle CaP attenuated contraction. However, when phosphorylated CaP was applied to the same preparations no effect was observed (Horowitz *et al.*, 1996) (fig 2).

Caldesmon is another actin binding protein that has been implicated in smooth muscle contraction (Hemric and Chalovich, 1988 ; Adam *et al.*, 1992). Studies have shown that caldesmon is able to bind both actin and myosin, and in the process attenuates contraction by inhibiting ATPase activity of the myosin head (fig 2). Like calponin, caldesmon-mediated inhibition of contraction is also abolished by phosphorylation.

While caldesmon is phosphorylated by PKC *in vitro* the only kinase which appears to phosphorylate it *in vivo* is the extracellular signal-regulated kinase (ERK), the final enzyme in the mitogen activated protein kinase (MAPK) cascade (fig 2). Activation of this pathway is typically triggered by mitogens, including growth factors and some agonists of G-protein coupled receptors (GPCR), that initiate tyrosine phosphorylation cascades ending in ERK activation (Schlessinger and Ullrich, 1992 ; Luttrell *et al.*, 1999). Activation of the MAPK cascade modifies gene transcription and stimulates cell proliferation. The observation that activated ERK phosphorylates caldesmon implies that, in addition to stimulation of mitosis, this pathway may also mediate smooth muscle contraction.

Activation of many GPCR is known to stimulate tyrosine phosphorylation in vascular smooth muscle. The observation that phorbol esters mimic the tyrosine phosphorylation induced by GPCR in vascular smooth muscle suggests that these receptors mediate tyrosine phosphorylation through a PKC dependent mechanism (Tsuda *et al.*, 1991). Activation of PKC may lead to the phosphorylation and activation of Raf-1, a serine/threonine kinase (mitogen activated protein kinase kinase kinase), which, in turn, triggers a signaling cascade leading to the phosphorylation and activation of the ERKs (Luttrell *et al.*, 1999). In light of these observations it has been suggested that PKC may induce contraction via activation of the MAPK pathway and subsequent phosphorylation of caldesmon.

1.3.1 PKC IN CEREBRAL VASOSPASM

It is likely that some of the mechanisms which maintain tone during physiological contraction are also involved in the pathological contraction observed following SAH. It has been speculated that PKC, an enzyme known to be involved in physiological tone maintenance, may also be a critical component of cerebral vasospasm following SAH, and numerous studies have provided support for this possibility. Intracisternal injections of phorbol esters are known to produce vasospasm in an *in vivo* canine model, indicating that activation of PKC is sufficient to initiate vasospasm (Sako *et al.*, 1993). In addition, levels of the endogenous activator of PKC, DAG, are reported to increase progressively in cerebral vessels after SAH, an effect which could conceivably lead to prolonged activation of the enzyme (Matsui *et al.*, 1990). Consistent with this observation, the turnover of PC is markedly increased in spastic arteries while that of phosphatidylinositol is unchanged, suggesting the sustained production of DAG through the hydrolysis of PC (fig 3)(Takuwa *et al.*, 1993). Moreover, PKC activity within the membrane fractions is significantly elevated during vasospasm in the canine SAH model (Sako *et al.*, 1993). Furthermore, inhibitors of PKC such as H7 and staurosporine have been shown to ameliorate vasospasm in an animal model (Matsui et al., 1991). While this evidence directly implies that PKC is involved in vasospasm it is now recognized that H7 and staurosporine are non-specific inhibitors of many protein kinases including MLCK. Therefore the conclusion that PKC is responsible for vasospasm cannot be completely supported by this evidence.

The involvement of the thin filament associated proteins, CaP and CaD, has also been investigated in the pathogenesis of vasospasm. It has been demonstrated that the

immunoreactivity of both CaP and CaD is significantly decreased in animal models of vasospasm, thus suggesting that proteolysis of these proteins may occur during vasospasm (Doi *et al.*, 1993). Degradation of these smooth muscle regulatory proteins would invariably lead to a loss of their inhibitory potential, and would thereby enhance the contractility of the spastic arteries (fig 3). The process of intracellular protein degradation may be due to the action of calpain, a calcium dependent protease. There is evidence that calpain levels are increased in vasospastic arteries (Minami *et al.*, 1992) and the levels of calpastatin, the endogenous inhibitor of calpain, are reduced (Yamaura *et al.*, 1993). These observations support a potential involvement of calpain mediated proteolysis of intracellular proteins in the pathogenesis of vasospasm.

In addition to cleavage of thin filament-associated proteins it has been recognized that calpain may enhance vasospasm by other mechanisms. Activated calpain may lead to the limited proteolysis of PKC isoforms. Calpain has been shown to cleave PKC in the region between the regulatory and catalytic domain (fig 3). This cleavage relieves the inhibitory constraint imposed by the pseudosubstrate (Nishizuka *et al.*, 1992), which results in uncontrollable phosphorylation of PKC target proteins, a process which may contribute to many pathological events occurring during vasospasm. Calpain may also posses the ability to cleave calcium pumps and channels which would contribute to the loss of calcium homeostasis and the accumulation of intracellular Ca^{2+}. This effect of calpain would be consistent with the prolonged calcium elevation observed following SAH.

1.3.2 ROLE OF Ca^{2+} IN CEREBRAL VASOSPASM

Calcium channels have been proposed to play a role in vasospasm following SAH. It has been reported that, during vasospasm and following smooth muscle cell exposure to OxyHb, there is a sustained elevation of $[Ca^{2+}]_i$ (Takanashi *et al.*, 1992 ; Vollrath *et al.*, 1994). In 1979 nifedipine, a dihydropyridine L-type calcium channel antagonist, was reported to prevent vasospasm (Allan and Bahr,1979). Several years later a clinical trial conducted by Allan *et al* (1983) demonstrated that nimodipine, a member of the dihydropyridine class of calcium channel antagonists, significantly reduced the severity of ischemic neurological deficit in patients with SAH. Thus, it was proposed that antagonism of voltage-dependent L-type calcium channels could reverse the vasoconstriction by limiting calcium influx into the vascular smooth muscle. Despite this evidence studies using the primate models of vasospasm consistently failed to show that calcium channel antagonists prevented vasospasm (Espinosa *et al.*, 1984 ; Nosko *et al.*, 1985). It has now been accepted that influx of calcium through voltage-gated calcium channels does not play any critical role in either the initiation or maintenance of vasospasm (Pickard *et al.*, 1989). However calcium channel blockers remain part of the standard treatment of vasospasm due to their well documented neuroprotective effects (Warnell, 1994). It is now hypothesized that sustained elevations in intracellular calcium concentrations, following exposure to OxyHb, may be due to the impairment of calcium homeostatic mechanisms. There is evidence that the function of the Ca^{2+}-Mg^{2+} ATPase, a calcium pump in the plasma membrane, is inhibited during vasospasm, an effect which would lead to intracellular calcium accumulation (Wang *et al.*, 1994)(fig 3). This calcium pump inhibition was shown to be mediated by free radicals and lipid peroxide

formation, which was reversed after administration of antioxidant aminosteroids (Rohn *et al.*, 1996). These observations suggest that OxyHb mediated free radical production and subsequent cellular damage leads to a sustained pathological elevation of the $[Ca^{2+}]_i$.

Given the importance of the free intracellular calcium concentration in smooth muscle contraction, the prolonged elevation of $[Ca^{2+}]_i$ observed during vasospasm would have a substantial impact on vascular contraction. If the sustained elevation does contribute to the persistent vascular constriction observed in vasospasm, then antagonists of intracellular calcium might prove useful. HA 1077, which was originally reported as an intracellular calcium antagonist, reduces vasospasm in a canine model (Shibuya *et al.*,1992 ; Takayasu *et al.*, 1986). Further investigation has found that HA 1077 has multiple non-specific effects including inhibition of MLCK and PKC. Therefore, any beneficial effects of this compound observed following vasospasm cannot be solely attributed to inhibition of intracellular calcium increases. Thus, while elevated intracellular free calcium concentration associated with vasospasm would contribute to the pathogenesis of the disorder, an attempt to alter these increases is not likely to prove fruitful due to the multifactorial nature of the condition.

1.3.3 Summary

At present it is clear that there are two key pathological processes which lead to vasospasm following SAH, 1) sustained elevation of intracellular calcium and 2) prolonged activation of PKC (fig 3). Unfortunately the current therapy of vasospasm fails to inhibit effectively either of these processes. Therefore it is not surprising that vasospasm following SAH is characterized by a high level of morbidity and mortality.

Due to the multifactorial nature of this disorder, successful treatment cannot lie with an agent capable of affecting only one specific mechanism; rather effective therapy should be sought with an agent that displays multifunctional properties against the pathological processes of vasospasm. Two possible candidates with the aforementioned features are the antiestrogen compound tamoxifen and polycationic aminoglycoside antibiotics, both of which are now recognized to be PKC, calcium, and free radical antagonists.

1.4 VASORELAXANT COMPOUNDS

1.4.1 TAMOXIFEN

Tamoxifen (TAM) is a nonsteroidal antiestrogen which has been shown to bind with high affinity to estrogen receptors. As a result, TAM is effective in the treatment of estrogen receptor positive breast cancers, and is being assessed as a preventative agent for this disease (Friedman, 1998). There is evidence which demonstrates that TAM is effective against estrogen receptor negative cancers (Gibson *et al.*, 1990). Therefore, while the ability of TAM antagonize estrogen receptors is well documented, its effects cannot be explained solely by estrogen receptor blockade (Friedman, 1998). It is now recognized that, in addition to action on estradiol receptors, TAM has numerous sites of action and binds to sites distinct from estrogen receptors and exhibits multiple effects (Aitken *et al.*, 1985). Some of the effects of TAM include inhibition of PKC (Cheng *et al.*, 1998 ; Horgan *et al.*, 1986 ; O'Brian *et al.*, 1985), antagonism of calmodulin and MLCK (Lam, 1984 ; Lopes *et al.*, 1990), and antioxidant activity (Wiseman, 1994).

It has been proposed that the ability of TAM to inhibit PKC may be mediated through two independent mechanisms. The first is a direct interaction with binding sites on the regulatory subunits of the enzyme, an interaction which prevents enzyme activation (O'Brian *et al.*, 1988). The second mechanism involves a disruption of the hydrophobic interactions between the regulatory subunit of PKC and phosphatidylserine, a lipid cofactor required for activation. TAM is believed to interact with membrane phospholipids and decrease membrane fluidity. It is thought that this alteration of membrane fluidity negatively modifies the binding capacity of proteins to membranes. Thus TAM may potentially mediate an inhibition of PKC activity by preventing the enzyme from binding to lipid co-factors required for its activation (Nakadate *et al.*, 1988).

The initial studies utilizing a crude brain extract in an isolated enzyme assay indicated that a high concentration of TAM (10-100 μM) is required for PKC inhibition (O'Brian *et al.*, 1988). However, subsequent studies examining the ability of TAM to attenuate PKC membrane translocation in intact cells, reported a significantly lower and clinically relevant concentration of TAM (1-10 μM) is sufficient (Wickman and Vollrath, 2000). Therefore, it is likely that the effectiveness of TAM arises from inhibition of PKC translocation and hence activation, which may help to explain the effectiveness of this compound in estrogen receptor negative cancers. The inhibitory action of TAM against PKC appears to be an important component of the action of this compound in the treatment of brain gliomas.

The binding of TAM to calmodulin has been examined in detail and two binding sites have been discovered (Edward *et al.*, 1992). TAM and analogues have been shown

to inhibit competitively the cellular effects of calmodulin, thus suggesting that TAM, when bound, inhibits the ability of calmodulin to recognize, bind, and activate its targets (Lam, 1984). It has been speculated that this antagonism of CaM by TAM and subsequent inhibition of downstream effectors, such as MLCK, might be one of the mechanisms responsible for the estrogen-independent pharmacological activities of the drug (Macgregor and Jordan, 1998).

TAM has a documented ability to protect membranes from oxidative damage. It has been shown that TAM and structurally related compounds, including estradiol, inhibit metal-ion-dependent lipid peroxidation of natural and model membranes (Wiseman *et al.*, 1990 and 1993). These compounds display lipophilic properties and are believed to insert in the membrane and decrease membrane fluidity (Clarke *et al.*, 1990). There has been a good positive correlation observed between decreased membrane fluidity and antioxidant ability, suggesting that inhibition of lipid peroxidation by TAM results from membrane stabilizing interactions (Wiseman *et al.*, 1992).

It is clear that TAM is capable of mediating many cellular effects in addition to its well-defined ability to antagonize estrogen receptors. As a result it has been realized that TAM may be of potential value in many pathological conditions. In particular, TAM mediates a relaxant effect on vascular smooth muscle and be of potential benefit for cerebral vasospasm (Wickman and Vollrath, 2000). Due to the multifactorial nature of vasospasm it has been suggested that a compound with multiplicity of actions may be required to treat this disorder effectively (Findlay *et al.*, 1991). TAM appears to be a good candidate as many of its effects may attenuate pathological processes occurring during vasospasm. Inhibition of CaM by TAM would mediate a pronounced relaxant

effect of vascular smooth muscle by limiting the activation of MLCK, an enzyme critical to smooth muscle contraction. In addition, the antioxidant properties of TAM may serve to protect the membrane against lipid peroxidation due to OxyHb auto-oxidation and free radical production, which would attenuate the initiation of smooth muscle contraction associated with vasospasm. Furthermore, inhibition of PKC may be a primary mechanism whereby TAM attenuates vasospasm induced by SAH. Thus, due to its multiplicity of actions TAM may succeed where many other therapies have failed and effectively attenuate or prevent cerebral vasospasm.

1.4.2 AMINOGLYCOSIDE ANTIBIOTICS

The aminoglycoside compounds constitute a group of antibiotics effective against gram negative bacilli. An important property of this group of compounds is their highly polar polycationic structure at physiological pH, which appears to be responsible for their ability to interact with anionic molecules in plasma membranes, such as the phosphate head groups of the acidic phospholipid, phosphatidylinositol (Marche *et al.*, 1983 ; Sastrasinh *et al.*, 1982). The binding of aminoglycosides to PIP_2 is recognized to have profound cellular effects which are independent of their bactericidal action (Marche *et al.*, 1983 ; Schibeci and Schacht., 1977). Neomycin and related aminoglycosides have been reported to inhibit PLC activity. This action is believed to be due to PIP_2 binding and has been widely reported in a variety of tissues and cells including vascular smooth muscle (Carney *et al.*, 1985 ; Hostetler *et al.*, 1982 ; Vollrath *et al.*, 1990). As a result of PLC inhibition, the production of the critical second messengers, IP_3 and DAG, would be

attenuated, a process which would in turn limit intracellular calcium release from the SR and restrict the activation of PKC isoforms.

A number of studies have shown that in addition to PLC, neomycin and other aminoglycoside antibiotics interfere with other signaling pathways leading to PKC activation which may explain the effectiveness of these compounds to relax smooth muscle contraction induced by OxyHb and ET-1. The ability of the aminoglycoside antibiotics to attenuate the activation of PKC may be due to inhibition of PLD which hydrolyses PC to phosphatidic acid (PA) and DAG, and activator of PKC (Rumenapp *et al.*, 1997). The aminoglycosides may also bind phosphatidylserine, a lipid co-activator of PKC (table 1), thus mediating a direct inhibition of PKC activation (Huang *et al.*, 1999). Consistent with this possibility, neomycin and several other aminoglycoside antibiotics are effective antagonists of PKC activity in purified preparations from rat brain (Hagiwara *et al.*, 1988). In addition, the aminoglycoside antibiotics reverse the contractile effects of phorbol esters, suggesting that these compounds mediate a direct inhibition of PKC (Nessim, 1997).. Hence, it appears that the aminoglycoside antibiotics are capable of inhibiting PKC activity by different mechanisms, an effect which may explain their vasorelaxant properties.

The aminoglycoside antibiotics may also possess an antioxidant action. Spermine, a polyamine similar to the aminoglycosides, has been shown to protect against fatty acid-induced lipid peroxidation in human breast cancer cells (Chapman and Wallace., 1994). Although, at present the antioxidant capacity of the aminoglycosides is not fully understood this feature of these compounds could further enhance their

therapeutic potential in the treatment of vasospasm as free radical formation is believed to be a central component in the pathogenesis of cerebral vasospasm.

Neomycin is also an effective inhibitor of KCl-induced contraction, which is dependent upon activation of L-type calcium channels and calcium influx for initiation of contraction (Nessim, 1997). This evidence suggests that, in addition to inhibition of PLC, the aminoglycosides may inhibit calcium flux as part of their relaxant mechanism. Consistent with this notion Adams *et al.* (1993) demonstrated that gentamicin antagonizes calcium influx and contractile function in vascular smooth muscle. As previously mentioned, neomycin was shown to attenuate both vasoconstriction and sustained intracellular calcium accumulation initiated by OxyHb, suggesting that mechanism of action of neomycin may be via the inhibition of Ca^{2+} influx (Vollrath *et al.*, 1994). While L-type channel inhibition by these compounds is not likely to reverse vasospasm this effect may limit neurotoxicity due to L-type channel activation and subsequent calcium overload. Therefore, the ability of the aminoglycoside antibiotics to inhibit cellular calcium flux may be of potential benefit in limiting cerebral vasospasm and its ischemic consequences following SAH.

Thus, it appears that the aminoglycoside antibiotics display a multiplicity of actions which may be of use in preventing or reversing vasospasm. The ability of the aminoglycosides to attenuate vasospasm was first studied in 1974, and at that time it was shown that oral kanamycin produced a significant improvement of angiographic spasm in a monkey model (Zervas *et al.*, 1974). These initial results led to a clinical trial of oral kanamycin which showed promising results (Zervas *et al.*, 1979). However, further studies using a monkey model cast doubts on the efficacy of oral kanamycin, which is

poorly absorbed from the gastrointestinal tract (Noseworthy *et al.*, 1984). Thus, the oral administration of aminoglycoside antibiotics for the treatment of cerebral vasospasm is not considered an effective therapy. Nonetheless, Nessim *et al* (1997) demonstrated the effectiveness of all of the aminoglycosides to produce a concentration-dependent relaxation of basilar artery rings preconstricted with either OxyHb or ET-1. It was hypothesized that the efficacy of these compounds is a result of multiple inhibitory actions on PLC, PKC, and intracellular calcium, some of the key processes thought to be involved in the pathogenesis of vasospasm. Thus, while these compounds may not be absorbed adequately or may not penetrate tissues sufficiently in order to be efficacious against vasospasm, a pellet implantation at the time of surgery would allow the aminoglycosides to exert their effects against cerebral vasospasm and may prove to be a useful compound in the treatment of this disorder.

1.5 HYPOTHESES AND OBJECTIVES

The ability of PKC to maintain prolonged vascular smooth muscle contraction has led to the proposal that this enzyme may be a mediator of cerebral vasospasm. Recent observations indicate that PKC may be activated in spastic cerebral arteries following SAH, which suggests that PKC is involved in the pathogenesis of the disorder. **Thus, we hypothesize that the activation of PKC by OxyHb and ET-1, the principal spasmogens implicated in the pathogenesis of cerebral vasospasm, plays a key role in the cerebral vasoconstriction produced by these compounds.**

The aminoglycoside antibiotics and tamoxifen have been proposed to interfere with many cellular pathways, such as the activation of PLC and PKC. Both pathways are thought to be involved in OxyHb and ET-1 stimulated contraction of vascular preparations. **As a result, we hypothesize that these compounds will reverse OxyHb and ET-1-induced contraction in cerebrovascular preparations.**

The objectives of the study are twofold. The first is to determine whether PKC is involved in the contractile response of cerebrovascular preparations to OxyHb and ET-1. The second is to determine the efficacy and the mechanism of action of both the aminoglycoside antibiotics and tamoxifen against vasoconstriction induced by OxyHb and ET-1.

To accomplish these experimental goals, we asked the following questions:

1. **Is the activation of PKC involved in the action of OxyHb and ET-1 on isolated cerebral artery preparations and cerebral vascular smooth muscle cells?**
2. **Are the effects of OxyHb and ET-1 on PKC activity time-dependent?**
3. **Which PKC isoforms are expressed in intact arteries and cultured cells?**

4. **Which of these isoforms are translocated to the membrane by stimulation with OxyHb and ET-1?**
5. **Which PKC isoforms are involved in the contractile responses to OxyHb and ET-1?**
6. **Do the aminoglycoside antibiotics or tamoxifen relax OxyHb and ET-1 induced contraction?**
7. **If so, then what is the relaxant mechanism of action of these compounds?**

Fig 1. Diagrammatic representation of iron dependent generation of oxygen free radicals in cell membranes. Oxyhemoglobin is autooxidized to methemoglobin and produces a superoxide radical that reacts with water (H_2O) to form hydrogen peroxide (H_2O_2). Hydrogen peroxide then reacts with ferrous iron to produce the hydroxyl radical which initiates the process of lipid peroxidation.

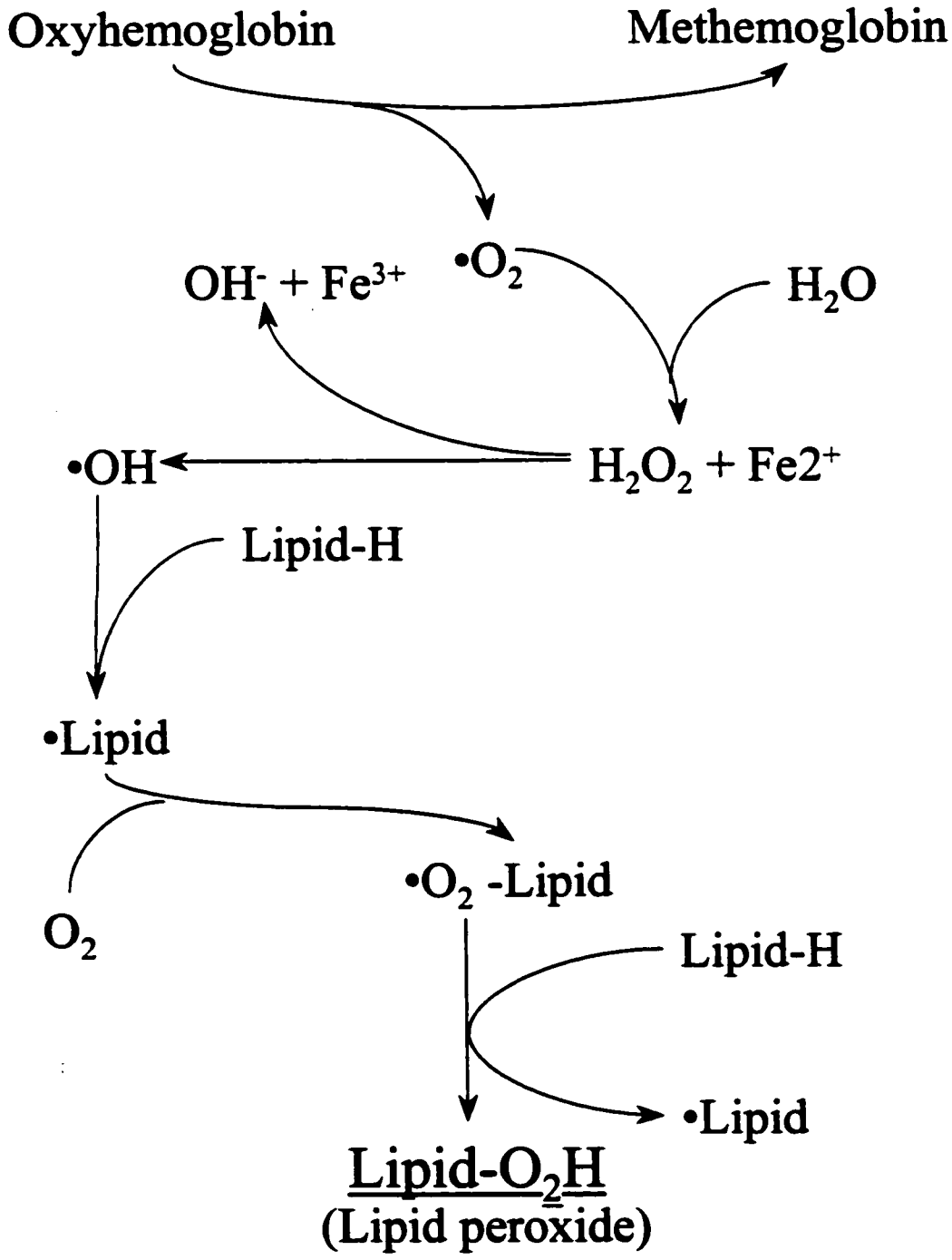

Oxyhemoglobin
Methemoglobin
$OH^- + Fe^{3+}$
$\bullet O_2$
H_2O
$H_2O_2 + Fe2^+$
•OH
Lipid-H
•Lipid
O_2
$\bullet O_2$ -Lipid
Lipid-H
•Lipid
Lipid-O_2H
(Lipid peroxide)

Fig 2. Diagrammatic representation of the signal transduction pathways leading to smooth muscle contraction. Agonist binding to receptor initiates activation of PLC-β via heterotrimeric G-protein. Activation of PLC leads to the hydrolysis of PIP_2 to DAG and IP_3. IP_3 triggers release of calcium from intracellular stores which binds to CaM and activates MLCK that, in turn, stimulates contraction. DAG activates PKC which phosphorylates intracellular proteins leading to contraction. Dashed lines indicate an indirect pathway. R, receptor; Gq, heterotrimeric GTP-binding protein; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; CaM, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; ATP, adenosine triphosphate; MAPK, mitogen activated protein kinase; P_i, inorganic phosphate.

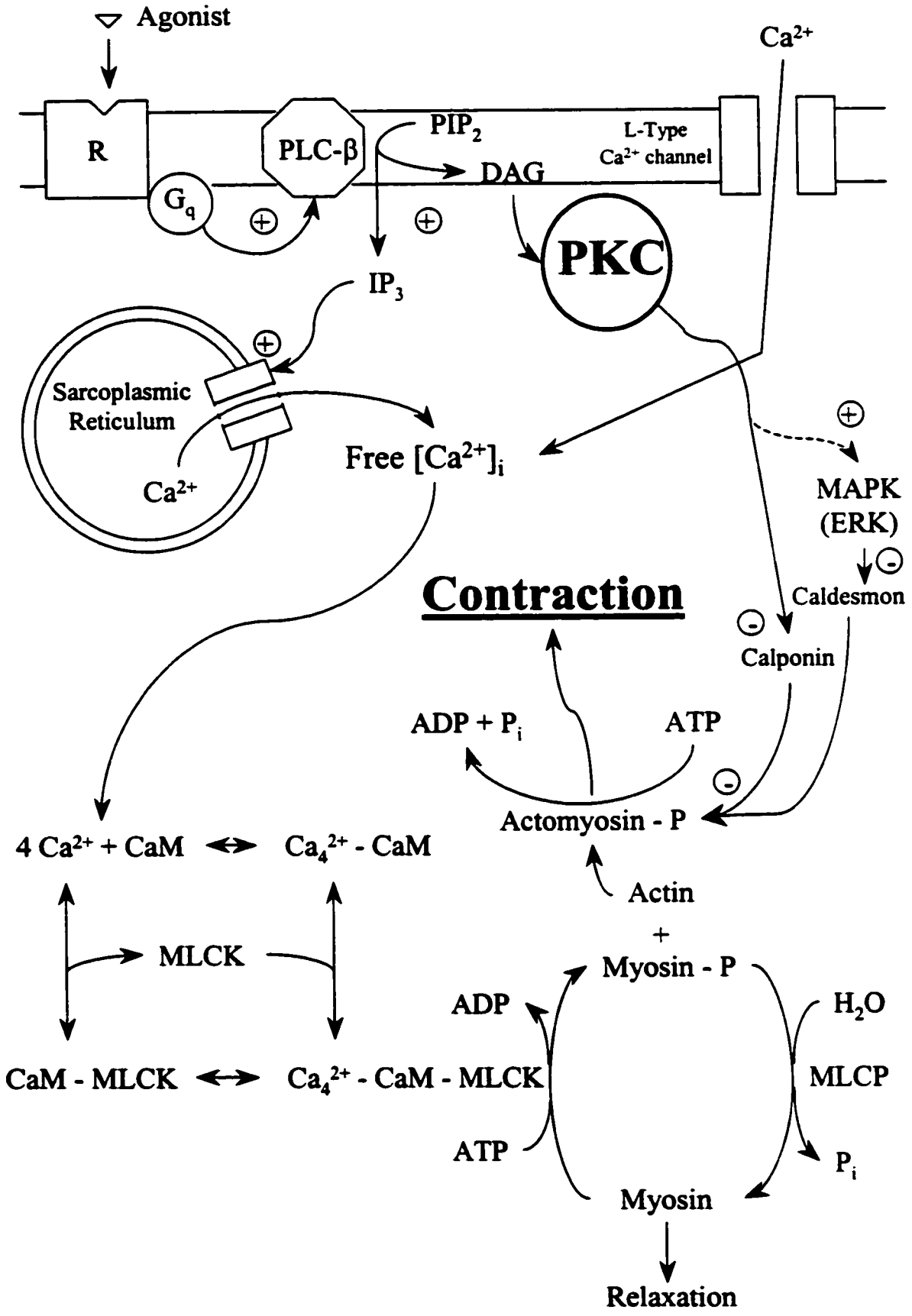

Agonist
Ca2+
R
PLC-β
PIP2
DAG
L-Type
Ca2+ channel
Gq
PKC
IP3
Sarcoplasmic
Reticulum
Ca2+
Free [Ca2+]i
MAPK
(ERK)
Caldesmon
Contraction
Calponin
ADP + Pi
ATP
Actomyosin - P
4 Ca2+ + CaM
Ca4 2+ - CaM
Actin
+
Myosin - P
MLCK
ADP
H2O
MLCP
CaM - MLCK
Ca4 2+ - CaM - MLCK
ATP
Pi
Myosin
Relaxation

Fig 3. Diagrammatic representation of the processes likely to be involved in the production of cerebrovascular spasm after subarachnoid hemorrhage. ET-1 R, endothelin-1 receptor; Gq, heterotrimeric GTP-binding protein; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PLD, phospholipase D; OxyHb, oxyhemoglobin; PKC, protein kinase C; MAPK, mitogen activated protein kinase. Lysing erythrocytes within the subarachnoid clot release OxyHb which may stimulate the production of ET-1. OxyHb causes increases in the intracellular calcium concentration and activate PKC, which may initiate smooth muscle contraction leading to sustained vasospasm. Endothelin also contributes to smooth muscle contraction through the activation of PKC and elevation of intracellular calcium. Dashed lines indicate an indirect pathway.

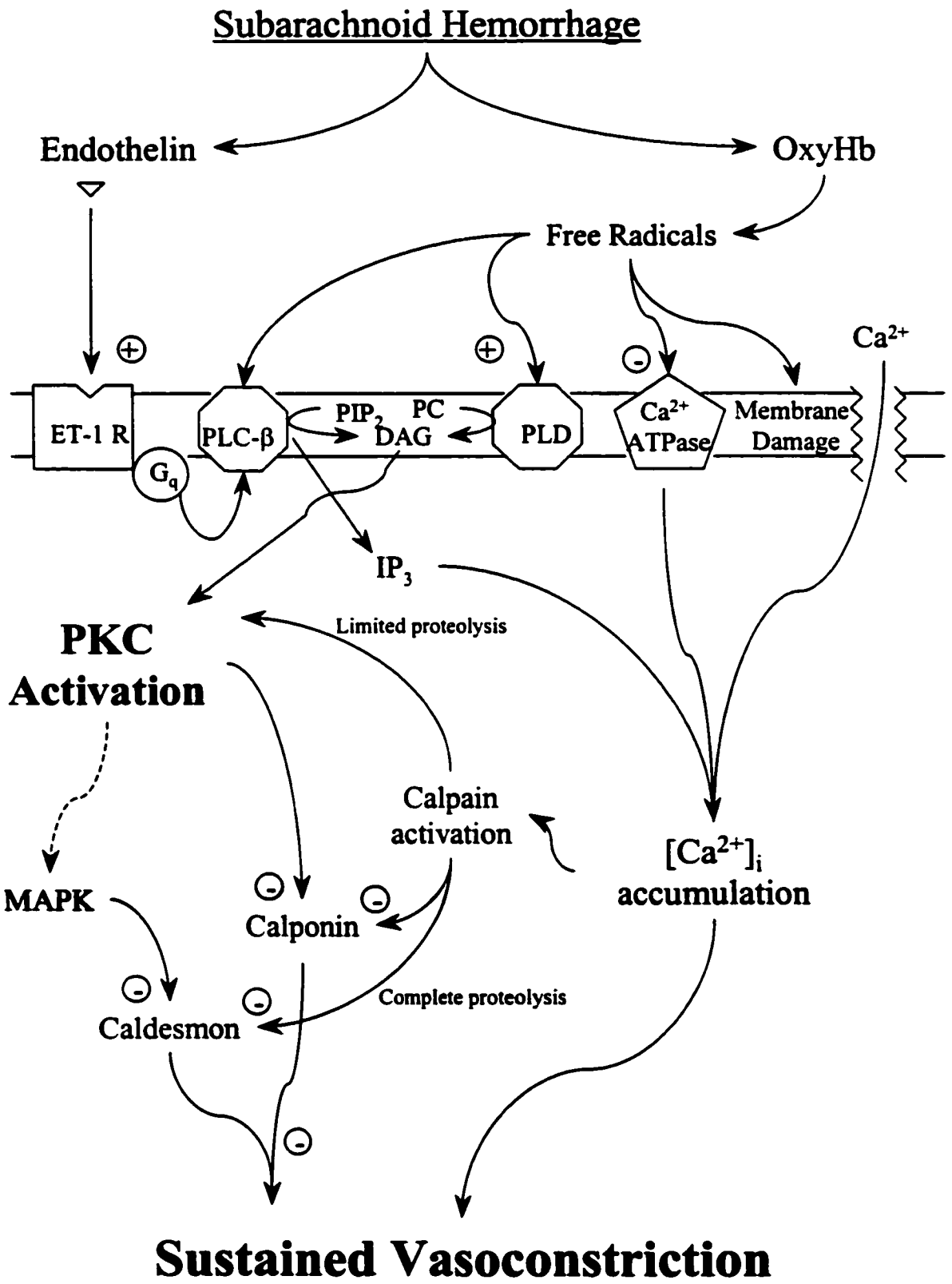

Subarachnoid Hemorrhage
Endothelin
OxyHb
Free Radicals
Ca2+
ET-1 R
Gq
PLC-β
PIP2
PC
DAG
PLD
Ca2+ ATPase
Membrane Damage
IP3
PKC Activation
Limited proteolysis
Calpain activation
[Ca2+]i accumulation
MAPK
Calponin
Caldesmon
Complete proteolysis
Sustained Vasoconstriction

Table 1. Subspecies of protein kinase C. Ca^{2+}, intracellular free calcium ions; PS, phosphatidylserine; DAG, diacylglycerol.

Subspecies	Isoform	Lipid Activators
Classical Protein Kinase C	α	Ca^{2+}, PS, DAG (Phorbol Ester)
	β_I/β_{II}	Ca^{2+}, PS, DAG (Phorbol Ester)
	γ	Ca^{2+}, PS, DAG (Phorbol Ester)
Novel Protein Kinase C	δ	PS, DAG (Phorbol Ester)
	ε	PS, DAG (Phorbol Ester)
	η	PS, DAG (Phorbol Ester)
	θ	PS, DAG (Phorbol Ester)
Atypical Protein Kinase C	ζ	PS
	λ	PS

CHAPTER 2:

2.0 MATERIALS AND METHODS

2.1 CONTRACTILITY EXPERIMENTS

The cerebral arteries were derived from New Zealand white rabbits (2.5-3.5 kg). Protocols for the humane treatment of animals according to the Declaration of Helsinki, and as approved by the University of Alberta Animal and Ethics review were followed in all experiments. The animals were anesthetized with an intravenous overdose of sodium pentobarbital (160 mg/kg), and the brain with the cerebral arteries were rapidly removed and placed in ice-cold Krebs-Henseleit solution (in mM: NaCl 130, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and dextrose 11). The basilar arteries were removed and cleaned of connective tissue, cut into 2-3 mm ring segments, denuded of endothelium and mounted using stainless steel hooks in organ baths containing Krebs-Henseleit buffer at 37°C, bubbled with 95% O_2 and 5% CO_2. The arterial rings were equilibrated under 1g of tension for 1h. The viability of the preparations was tested with 60 mM KCl, at the beginning of experiments. DMSO was used as a vehicle control for PMA and tamoxifen and was devoid of any vasoactive properties. All contractile responses were recorded isometrically using force-displacement transducers and a Grass 7D polygraph calibrated to 1 gram tension.

2.1.1 EXPERIMENTAL PROTOCOLS

2.1.1.1 EFFECTS OF ENDOTHELIUM

Isolated ring preparations were contracted with 5-HT (10 μM) and the responses to increasing concentrations of bradykinin (10^{-12}-10^{-7} M) were observed. Bradykinin is known to produce an endothelium dependent relaxation. Therefore, the absence of

endothelium was confirmed by the lack of a vasorelaxant response to bradykinin. The e-NOS inhibitor L-NAME (1 μM) was added to block NO production. No effect was observed thus confirming the absence of endothelium.

2.1.1.2 INVOLVEMENT OF ET RECEPTORS

Isolated rings of basilar arteries were exposed to increasing cumulative concentrations of ET-1 (10^{-11}-10^{-7} M) in order to generate concentration effect curves. The maximum contraction was determined when no further increase in tension was observed with an increased concentration. The preparations were immediately washed and when contraction returned to resting tension the arteries were exposed to BQ-123 (100 nM), an ET_A receptor antagonist, for 20-30 min. Following exposure to BQ-123 the tissues were re-exposed to increasing cumulative concentrations of ET-1 in order to determine which ET-1 receptor is involved in the vasoconstrictor response.

2.1.1.3 EFFECTS OF THE VASORELAXANT COMPOUNDS

The basilar artery rings were pre-constricted with agonists prior to vasorelaxation studies. The agonists used to constrict the vessels were OxyHb (10 μM), ET-1 (0.1 nM), or PMA (160 nM). When OxyHb was used, 50 μl of 1% aqueous emulsion of antifoam B was added to the organ bath to prevent foam formation by OxyHb. The PKC inhibitors, Ro32-0432 (56 and 360 μM) and Gö 6976 (5-100 nM), were added in cumulative concentrations during maximum developed contraction, in order to determine the involvement to PKC isoforms during sustained vascular tone due to various spasmogens. In a separate series of studies, the estrogen antagonist, tamoxifen (0.1-5

μM), and the aminoglycoside antibiotics (0.01-5 mM), gentamicin, neomycin, kanamycin, and streptomycin were cumulatively added to pre-contracted ring preparations to assess their vasorelaxant properties. The resting tension was used to define maximum relaxation and the tonic phase of contraction was considered as 100% constriction. IC_{50} values for these compounds were determined with a semi-log plot fitted to a sigmoidal curve with the program SlideWrite 3.0.

2.1.1.4 Effect of gentamicin and Ro32-0432 in calcium free medium

Basilar artery rings were equilibrated as described above. The Krebs-Henseleit buffer was then replaced by calcium-free Krebs-Henseleit solution containing 2 mM EGTA. Calcium depletion was confirmed by lack of response to 60 mM KCl. The vessels were pre-constricted with PMA (160 nM), and gentamicin (0.01-5 mM) or Ro32-0432 (360 nM) were added following maximal tension development.

2.2 Cell culture

Basilar arteries were isolated under sterile conditions and placed in a petri dish containing Dulbecco's modified Eagle medium (DMEM). The adventitia was mechanically removed and the vessels were cut into segments 5 mm in length along the longitudinal axis, and the endothelium was removed with gentle scraping (Takanashi *et al.*, 1992). The tissue was then chopped into 1-2 mm segments and placed in 25 cm^2 culture flasks containing 1 ml DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (10 μg/ml). When the primary cultures were nearly confluent the cells were transferred to 75 cm^2 flasks and then routinely subcultured at a

split ratio of 1:3. The cells tested positive for smooth muscle α-actin and at confluence the cells demonstrate the typical "hill and valley" pattern (Takanashi, *et al.*, 1992).

2.3 MEASUREMENTS OF PROTEIN KINASE C ACTIVITY

Protein kinase C (PKC) activity was measured in the membrane and cytosolic fractions of serum-starved (24 h) cerebrovascular smooth muscle cells stimulated by OxyHb (10μM), ET-1 (0.1 nM), and phorbol myristate acetate (PMA) (160 nM), at various times. After incubation the cells were washed with ice-cold phosphate buffered saline (145 mM NaCl, sodium phosphate 10 mM; pH 7.4) and then scraped and collected in the same solution. The cells were then centrifuged at 200 x g for 10 min, the supernatant was discarded and the pelleted cells were transferred to small hand held glass tissue grinders and homogenized in ice-cold homogenization buffer containing Tris-HCl (50 mM), NaCl (150 mM), NaF (40 mM), ethylenediaminetetraacetic acid (EDTA) (3.5 mM), ethyleneglycol-bis-N,N,N',N'-tetraacetic acid (EGTA) (3.5 mM), sodium orthovanadate (1 mM), phenylmethylsulfonyl flouride (PMSF) (.5 mM), leupeptin (20 μM), Nonidet P-40 (NP-40) (0.02%). The homogenate was partitioned into cytosolic and membrane fractions by centrifugation at 15 000 x g for 60 min at 4°C. The supernatent was removed and reserved as the soluble cytosolic fraction and the remaining membrane pellet was re-suspended by sonication in ice-cold homogenization buffer supplemented with 0.5% NP-40. This portion was reserved as the membrane fraction. Samples were adjusted to equivalent protein concentration using the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

Cellular fractions were then used in an enzymatic assay using a fluorescent peptide substrate for PKC. The enzymatic assay consisted of a standard reaction mixture containing 100 mM Tris at pH 7.4, 10 mM ATP, 50 mM $MgCl_2$, .5 mM $CaCl_2$, phosphatidylserine (PS) (1 mg/ml), and the peptide substrate. The sample containing PKC was added to this reaction mixture and incubated for 30 min at 30°C. The reaction mixture was then applied to separation units containing affinity membranes (Pierce), which specifically bind the phosphorylated peptide. The bound substrate was eluted from the affinity membranes using a buffer containing 15% formic acid, and its absorbance was measured at 570 nm. A standard curve was generated with purified PKC of a known specific activity of 0.02 U/μL, where a unit (U) of PKC activity is defined as the amount that will transfer 1 nmole of phosphate to histone H1 per minute at 30°C. The PKC standard was diluted to specific activities (0.02-0.00125 U) and subjected to the assay and absorbance readings were acquired for the samples. From this data a standard curve was generated with SlideWrite 3.0 to which all experimental samples were compared and activity values were obtained.

2.4 IMMUNOBLOTTING PROTOCOLS

Western blot was performed using canine cerebrovacular smooth muscle cell culture and intact basilar artery obtained from New Zealand white rabbits. As described previously, protein concentration of the samples was determined by the Bradford method using BSA as a standard and then adjusted to equivalent levels. Homogenized cell samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with stacking gel concentration of 4% and separating gel concentration of

10%. After gel electrophoresis (100 V for 90 min) the proteins were wet-transferred onto nitrocellulose (100 V for 1 h). In some experiments, Ponceau S staining was performed to confirm that an equal amount of protein was loaded in each lane. The nitrocellulose was incubated in a solution of Ponceau S for 5 min and then washed in distilled water for an additional 5 min. Staining of the nitrocellulose was then accomplished and the bands visualized which allowed for confirmation of equivalent protein loading across the lanes. The membranes were blocked in Tris buffered saline (20 mM Tris, 120 mM NaCl) containing 5 % non-fat milk for 1h with constant agitation. The blocked membranes were then incubated overnight in blocking solution containing the appropriate concentration of the primary antibody. The blots were then washed every 5 min for 25 min in TBS containing 0.1% Tween 20. The membranes were then incubated with goat anti-mouse or goat anti-rabbit horse radish peroxidase linked secondary antibodies in blocking solution at a dilution of 1:3000 and 1:40000, respectively. Proteins were then visualized with a ECL chemiluminescence detection system (Amersham) applied to the membranes for 1min then exposed in the dark room to Hyperfilm (Amersham) for 30-60 sec and developed with a kodak automatic developer. To quantify and compare levels of protein the films were subjected to densitometic analysis using an Epson perfection 636 scanner with Epson Twain scanning software. Following scanning the density of bands was measured using Sigma-Gel software (Jandel Corp., San Rafael, CA). Molecular weight of all proteins was estimated by using pre-stained protein markers (Bio-Rad).

2.4.1 EXPERIMENTAL PROTOCOLS

2.4.1.1 EXPRESSION OF PKC ISOFORMS IN CULTURED CELLS AND INTACT BASILAR ARTERIES

Cerebrovascular smooth muscle cells were serum-starved for 24 h, washed with ice-cold phosphate buffered saline (145 mM NaCl, sodium phosphate 10 mM; pH 7.4) and scraped and collected in the same solution. The cells were centrifuged at 200 x g for 10 min, the supernatant was discarded and the pelleted cells were transferred to small hand-held glass tissue grinders and homogenized in ice-cold homogenization buffer containing Tris-HCl (50 mM), NaCl (150 mM), NaF (40mM), EDTA (5 mM), EGTA (5 mM), sodium orthovanadate (1mM), PMSF (0.5 mM), leupeptin (20 μM), NP-40 (0.02%). For intact basilar arteries, the samples were rapidly frozen by placement in a cryovial cooled with liquid nitrogen and stored until use. When ready to be applied to the western blot the frozen arteries were transferred to chilled plastic vials with homogenization buffer. The arteries were finely chopped with iris scissors and sonicated to ensure complete homogenization. Cerebrovascular smooth muscle cells (CVSMC) or quick frozen basilar artery homogenates were subjected to the western blotting procedure. Following blotting and Ponceau staining the lanes were marked and cut into ribbons, each ribbon was then blocked and exposed to different anti-PKC antibodies overnight. Antibodies directed against the α,γ, and ε isoforms of PKC were used at a 1:1000 dilution, the antibodies against PKC β,θ, and λ were used at 1:250 dilution, and the anti-PKC δ antibody was used with a 1:500 dilution. In the case of intact basilar arteries the nitrocellulose ribbons were probed twice with different PKC antibodies. Following the

first exposure of the antibodies were stripped with NaOH (0.2 M) for 5 min then washed with distilled water, the ribbons were then re-probed with antibodies for different PKC isoenzymes.

2.4.1.2 MEASUREMENT OF PKC TRANSLOCATION

Cerebrovascular smooth muscle cells were serum-starved for 24 h then treated with the agents in question for the appropriate time. The samples of intact basilar arteries were dissected and rapidly frozen. The samples were homogenized as described above. The homogenate of either cells or arteries was separated into cytosolic and membrane fractions by centrifugation at 15 000 x g for 60 min at 4°C. The supernatant was removed and reserved as the soluble cytosolic fraction and the remaining membrane pellet was re-suspended by sonication in ice-cold homogenization buffer supplemented with NP-40 (1%), sodium deoxycholic acid (0.1%), SDS (0.1%). These homogenates were immunoblotted as previously described. All antibody dilutions were as described above.

2.5 MATERIALS

Hemoglobin, tamoxifen, phorbol 12-myristate 13-acetate (PMA), L-NAME were purchased from Sigma. ET-1, Ro32-0432, and Gö 6976 were from Calbiochem. BQ-123 was from RBI. All reagents for colorimetric protein kinase C assay were from Pierce. OxyHb was prepared by a modification of the method described by Martin *et al.*, 1985. In this procedure ferric MetHb is reduced using sodium dithionate (10 mM) in a solution of 1 mM hemoglobin, the reducing agent is then removed by extensive dialysis, using a

1000 volume of 0.9% NaCl at a temperature of 2-8 C for 6h. The purity of OxyHb was then assessed by absorption spectrophotometry. The dialyzed solution of OxyHb was aliquoted and stored under liquid nitrogen.

2.6 STATISTICAL METHODS

All results are reported as the mean ± s.e.m., with the numbers of preparations used in parentheses. Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnet's test when significant probability was reached. Values of $P < 0.05$ were considered to be significant.

CHAPTER 3:

2.0 RESULTS

3.1 ROLE OF PKC IN CEREBROVASCULAR CONTRACTION

3.1.1 CONTRACTILITY STUDIES

3.1.1.1 EFFECTS OF OXYHB, ET-1, AND PMA ON BASILAR ARTERY CONSTRICTION

The contractile effects of OxyHb and ET-1 on basilar arteries were examined and are shown in fig 4A and fig 4B. OxyHb administered at a single concentration (10μM) which has been shown to be present in the CSF of patients following SAH (Pluta *et al.*, 1988) produced a slowly developing sustained contraction (fig 4A). Furthermore, OxyHb induced vasoconstriction in the presence of the NOS inhibitor, L-NAME (1μM), suggesting that the action of this spasmogen was endothelium independent. As shown in fig 4B, endothelin was also a potent contractile agonist of cerebral arteries. When administered in a single concentration (0.1nM) this agent produced a rapidly developing sustained contraction. In addition, ET-1 induced a concentration-dependent contraction of cerebral artery preparations with an EC_{50} of 0.48 nM, a concentration similar to that detected in the CSF of patients with SAH, as shown in fig 5.

To elucidate the endothelin receptor subtype responsible for the ET-1-induced contraction, the responses to ET-1 were examined in the presence or absence of the selective ET_A receptor antagonist, BQ-123, administered at its IC_{50} concentration (0.1 μM). As shown in fig 5, exposure of vascular preparations to BQ-123 resulted in a competitive antagonism of the ET-1 responses. Higher concentration of this antagonist (1 μM) abolished the responses to ET-1 (results not shown). Together these observations

indicate that the vasoconstriction caused by ET-1 in cerebral artery preparations was mediated predominantly by activation of the ET_A receptor subtype.

In order to examine whether PKC plays a role in vascular smooth muscle contraction, phorbol myristate acetate (PMA), a potent activator of PKC isoforms, was administered in a concentration of 160 nM, a dose selective for PKC activation (Nishizuka, 1995). This compound initiated a slowly developing powerful sustained contraction of isolated basilar arteries, suggesting that activation of PKC is sufficient to initiate and maintain vascular contraction (fig 4C). In contrast, the exposure of the preparations to 4α-Phorbol (320nM), an inactive phorbol ester analogue, did not produce any contractile effect, thus indicating that PMA-induced contraction occurs via activation of PKC. Furthermore, the contractions to OxyHb, ET-1, and PMA were resistant to relaxation, and only ET-1 induced contraction could be effectively relaxed with extensive washing (results not shown).

3.1.1.2 EFFECT OF ENDOTHELIUM REMOVAL ON BRADYKININ-INDUCED RELAXATION OF BASILAR ARTERIES

Generation of NO by the vascular endothelium results in a relaxant effect on smooth muscle. Therefore, it is critical to confirm the absence of endothelium in order to indicate whether the effects of the vasorelaxants are mediated in an endothelium-independent manner. We accomplished this pharmacologically by evaluating the responses to increasing cumulative concentrations of bradykinin (10^{-12} M - 10^{-7} M), in preparations which had been pre-contracted with 10μM 5-hydroxytryptamine. Bradykinin is known to produce endothelium-dependent relaxation which is not seen

when the endothelium is removed (Gergawy *et al.*, 1998). A representative response to bradykinin is shown in fig 6. In these experiments no relaxant effects were observed after administration of bradykinin, and contraction resulted at the highest concentration of this agent, confirming that the endothelium was removed. In another series of experiments vascular preparations were pre-incubated with an inhibitor of NOS, NG-nitro-L-arginine-methylester (L-NAME) (10^{-4}) for 30 min prior to addition of OxyHb, ET-1, or PMA (results not shown). The presence of L-NAME did not significantly effect the contractions induced by either agonist, confirming that NO release was not a factor effecting vasoconstriction.

3.1.1.3 Effect of the PKC inhibitor, Ro32-0432, on OxyHb, ET-1, and PMA induced contraction

To examine further the involvement of PKC in contractions induced by OxyHb and ET-1, the PKC inhibitor, Ro32-0432, was administered to vascular preparations which had developed contraction to these vasoconstrictors. Ro32-0432 is a specific, cell permeable inhibitor of PKC isoforms, and displays a 10-fold greater selectivity for classic PKC isoforms (PKC α and PKC β)(IC_{50}=28nM) over PKC ε (IC_{50}=180nM) (Wilkenson *et al.*, 1993 ; Way *et al.*, 2000). Ro32-0432 administered to the preparations, after tonic contraction to OxyHb, ET-1, or PMA had developed, produced a concentration-dependent relaxation. Representative traces are shown in fig 7A, 7B, and 7C. As shown in fig 8, Ro32-0432 (56 nM), at a concentration which inhibits the classic isoforms of PKC, produced 43 ± 13%, 59 ± 12%, and 23 ± 6% relaxation of tonic contraction induced by OxyHb, ET-1, and PMA, respectively. Inhibition of classical and novel (PKCε)

isoforms of PKC with Ro32-0432 (360 nM) initiated a highly significant relaxation of 89 ± 6% and 94 ± 3% for OxyHb and ET-1 induced contraction, respectively (fig 8). Ro32-0432 (360 nM) abolished contraction produced by PMA (160 nM) (fig 8). To test whether the ability of Ro32-0432 to relax contraction of cerebral artery preparations was due to inhibition of PKC the compound was tested against a PKC independent contraction induced with KCl. Preparations contracted with a depolarizing concentration of KCl (60 mM) were resistant to the relaxant effects of Ro32-0432 (results not shown), confirming that the relaxant properties of the Ro32-0432 is via inhibition of PKC isoforms.

3.1.1.4 Effect of the PKC inhibitor, Gö 6976, on OxyHb, ET-1, and PMA induced contraction

The involvement of the classical isoforms of PKC in vascular contraction induced by OxyHb and ET-1 was confirmed using the indocarbazole PKC inhibitor, Gö 6976. Gö 6976 is selective for the classical isoforms of PKC and inhibits the α and β isozymes with IC_{50} of 2.3 and 6.2 nM, respectively (Martiny-Baron *et al.*, 1993). This compound does not inhibit the kinase activity of the PKC ε isoform. As seen in fig 9A and 9B cumulative concentrations of Gö 6976 produced only incomplete relaxation of OxyHb and ET-1-induced tonic contraction, with values of 78 and 80 %, respectively. This observation suggests that, in addition to the classical PKC subtypes, PKCε may be involved in vasoconstriction mediated by these agents. Gö 6976 was less effective at inhibiting PMA-induced contraction (fig 9C), and induced 67% of relaxation with the

highest concentration (100 nM), indicating the contraction of cerebral arteries in response to PMA is via activation of PKCε.

3.1.2 Measurements of PKC activity

3.1.2.1 Time-course of the OxyHb and ET-1 effects on PKC activity

To explore further the involvement of PKC in the effects mediated by OxyHb and ET-1, direct measurements of PKC activity were performed using canine CVSMC. Since activated PKC translocates to the plasma and/or nuclear membranes, a process which is a hallmark of activation, the activities of the enzyme were determined in both cytosolic and membrane fractions of CVSMC. PKC activity in these samples was determined at the time points corresponding to the maximum tonic contraction. As shown in fig 10, exposure to OxyHb (10 μM), for 5 min, significantly increased PKC activity associated with the membrane fraction (228 ± 13% of control). The increase in PKC activity was maintained after 15 and 60 min exposure to OxyHb with values of 219 ± 13% and 183 ± 34% of control, respectively. This observation is consistent with PKC involvement in prolonged OxyHb stimulated smooth muscle contraction. PKC activity then declined (24 h) to levels which were not significantly different from control. The rise in membrane-bound PKC activity was associated with a concomitant decrease in the activity of this enzyme in the cytosolic fractions (32 ± 7%, 44 ± 6%, and 49 ± 9% at 5, 15, and 60 min respectively). At 24 hours, there was a large increase in the activity of PKC in the cytosolic fraction. The time course of ET-1-induced increases in membrane associated PKC activity is shown in fig 11. Five min exposure of smooth muscle cells to ET-1 (0.5

nM) increased PKC activity in the membrane fraction by 202 ± 17% of control. The increase in PKC activity was also observed after 60 min (281 ± 30% of control). There were corresponding decreases in the cytosolic fraction in cells treated with ET-1, suggesting that ET-1 stimulated translocation of PKC.

3.1.2.2 TIME-COURSE OF PMA EFFECTS ON PKC ACTIVITY

PMA (160 nM), an activator of PKC, was used as a positive control in order to confirm that PKC activation results in increased membrane-associated PKC activity. As shown in fig 12, exposure of the cells to PMA significantly increased membrane associated PKC (205 ± 17% of control values) following 5 min exposure. PMA was also a potent stimulator of PKC translocation at 15 and 60 min (206 ± 14% and 354 ± 105% of control, respectively).

3.1.3 WESTERN BLOT STUDIES

3.1.3.1 ANALYSIS OF PKC ISOFORM EXPRESSION

PKC represents a family of 13 isoforms and in order to determine which isoforms are present in cerebrovascular preparations an analysis of protein expression was performed. Western blots of cultured CVSMC and intact basilar arteries were probed with antibodies directed against multiple PKC isoforms and the results are shown in fig 13A and 13B. The cultured cells displayed immunoreactivity towards the α, δ, ε, and λ isoforms of PKC and failed to show expression of the β, γ, and θ isozymes. Intact basilar arteries expressed PKC α, β, ε, and λ while δ, and θ were not found. Identification of

these PKC isoforms was confirmed using positive controls and molecular weight markers.

3.1.3.2 Studies of PKCα and PKCε translocation induced by OxyHb and PMA

PKCα and ε are two isoforms which are consistently expressed in CVSMC and whole arteries, and both isoforms have been implicated in vascular contraction. Thus, in order to examine whether these two PKC isoforms may be involved in contraction of cerebral vessels we have examined the ability of OxyHb to induce translocation, and hence activation of these enzymes, in cultured CVSMC. Translocation of specific PKC isoforms was determined by assessing the PKC expression in cytosolic and membrane fractions of cultured CVSMC and subsequent densitometry analysis. The effects of OxyHb and PMA on translocation of the PKC isoforms were examined at the time point (60 min) corresponding to maximum tonic contraction.

A representative western blot demonstrating the translocation of PKC α is shown in fig 14A. Exposure of CVSMC to OxyHb (10 μM) and PMA (160 nM) significantly increased membrane associated PKC α (310 ± 45% and 255 ± 38%, respectively) as determined by densitometry analysis (fig 14B).

The ability of OxyHb (10μM) and PMA (160 nM) to stimulate PKCε translocation is shown in fig 15A. A 60 min exposure to OxyHb and PMA induced a 133 ± 13% and 150 ± 3% (fig 15B) increase in membrane-bound PKCε, compared with control values.

3.2 RELAXANT EFFECTS OF TAMOXIFEN

3.2.1 CONTRACTILITY STUDIES

The nonsteroidal antiestrogen TAM possesses antioxidant activity and has been reported to be an inhibitor of PKC, PLC, MLCK, and CaM (Wiseman, 1994). Each of these effects of TAM may be of potential benefit in reversing vasospasm. Therefore we have examined the ability of TAM to inhibit OxyHb, ET-1, and PMA induced vascular constriction in order to evaluate its potential as a therapeutic agent for cerebral vasospasm and to determine its mechanism of action. To mimic the situation in vasospasm, in which therapeutic agents are administered after vasospasm had been initiated, TAM was added to basilar artery preparations in which a tonic contraction had developed. fig 16 A, B, and C show representative traces of basilar artery rings contracted with OxyHb (10 μM), ET-1 (50 nM), or PMA (160 nM). Since we have established that activation of PKC isoforms may be involved in sustained contraction to the spasmogens we hypothesized that the ability of TAM to relax contraction may be attributed to inhibition of PKC. Thus, we have examined efficacy of this agent against PMA (160 nM) induced contraction. Increasing cumulative concentrations of tamoxifen (0.1 μM- 5 μM) abolished the contraction induced by OxyHb, ET-1, or PMA and had an IC_{50} value of 0.66 ± 0.09, 0.35 ± 0.01, and 1.1 ± 0.1 μM, respectively (fig 17).

To test whether the ability of TAM to relax vascular preparations is independent of its anti-estrogenic effects, the estradiol receptor agonist, 17β-estradiol, was used. In these experiments cerebrovascular ring preparations were pre-contracted with OxyHb and exposed to increasing cumulative concentrations of 17β-estradiol (0.01 nM- 1μM). 17β-

estradiol was found to have no effect on the contractility and when increasing cumulative concentrations of TAM were added in the presence of estradiol no change in the relaxant properties of TAM was observed (results not shown). Treatment with TAM also attenuated responses to depolarizing concentrations of KCl (60 mM) with an IC_{50} of 0.28 ± 0.06 μM.

3.2.2 The effects of tamoxifen on PKC activity

To examine further the role of PKC inhibition as a mechanism of TAM mediated vascular relaxation, direct measurements of PKC activity were performed. As shown in fig 18 exposure of cultured CVSMC to OxyHb (10 μM) or PMA (160 nM) for 5 min significantly increased PKC activity in the membrane fraction (253 ± 7.7% and 224 ± 8.2%, respectively). However, when the CVSMC were exposed to TAM (7 μM) 30 min prior to treatment with OxyHb (10 μM) or PMA (160 nM), the PKC activity in the membrane fractions was not different from the control levels (fig 18). These observations suggest that the ability of TAM to inhibit vasoconstriction is due, at least in part, to the inhibition of PKC activity.

3.3 Relaxant effects of the aminoglycoside antibiotics

3.3.1 Contractility studies

It has been previously reported that the aminoglycoside antibiotics possess a relaxant effect against OxyHb and ET-1 induced contraction (Nessim, 1997). The relaxant effects of the aminoglycosides on basilar arteries was confirmed and shown in

fig 19. Increasing cumulative concentrations of the aminoglycoside antibiotic, gentamicin, relaxed tonic contraction induced by ET-1 (fig 19A) and PMA (fig 19B).

In order to clarify the importance of PKC inhibition in the relaxant properties of the aminoglycosides these compounds were tested against a calcium-independent contraction. The vasorelaxant ability of the aminoglycosides was tested against vasoconstriction induced by PMA (160 nM) in calcium-free medium supplemented with EDTA (5mM). As shown in fig 20, the PMA-induced contraction was maintained in calcium free buffer and a depolarizing concentration of KCl (60 mM) had no effect, confirming an absence of free calcium. This contraction was attenuated by gentamicin and Ro32-0432 administered after a plateau tension had developed, indicating that the relaxant effect of gentamicin was indeed the consequence of PKC inhibition and not due to decreased Ca^{2+} entry.

3.3.2 The effects of the aminoglycosides on PKC activity

The effects of the aminoglycosides on PKC were further examined by performing direct measurements of activity of this enzyme using CVSMC stimulated with ET-1 (0.5 nM) and PMA (160 nM). Activity was determined in both cytosolic and membrane fractions at the time corresponding to maximum tonic contraction. As shown in fig 21 A and B, ET-1 and PMA elevated PKC activity in the membrane fractions. The aminoglycoside antibiotics, gentamicin, neomycin, kanamycin, and streptomycin, administered in the concentrations corresponding to the EC_{50} values determined from previous contractility studies, reduced the effects of ET-1 on PKC activity in the membrane fractions to 111 ± 11%, 107 ± 13%, 122 ± 15%, and 129 ± 12% of control,

respectively (fig 21A). The effects of PMA on PKC activity were significantly reduced to 103 ± 7%, 100 ± 9%, 114 ± 5%, and 88 ± 4% of control by gentamicin, neomycin, kanamycin, and streptomycin, respectively (fig 21B). These results are consistent with the hypothesis that vascular relaxation induced by the aminoglycosides is associated with the inhibition of PKC activity in CVSMC.

Fig 4. Contractile effects of OxyHb, ET-1, and PMA. Representative traces of the vasocontrictor responses induced by OxyHb (10 μM) (panel A), ET-1 (0.1 nM) (panel B), and PMA (160 nM) (panel C) on basilar artery rings. The nitric oxide synthase inhibitor, L-NAME, (1 μM), was added prior to OxyHb. The inactive phorbol ester, 4α-Phorbol (320 nM), was also administered prior to PMA. All responses were maintained for at least 90 min. Arrows denote time of administration of indicated compounds.

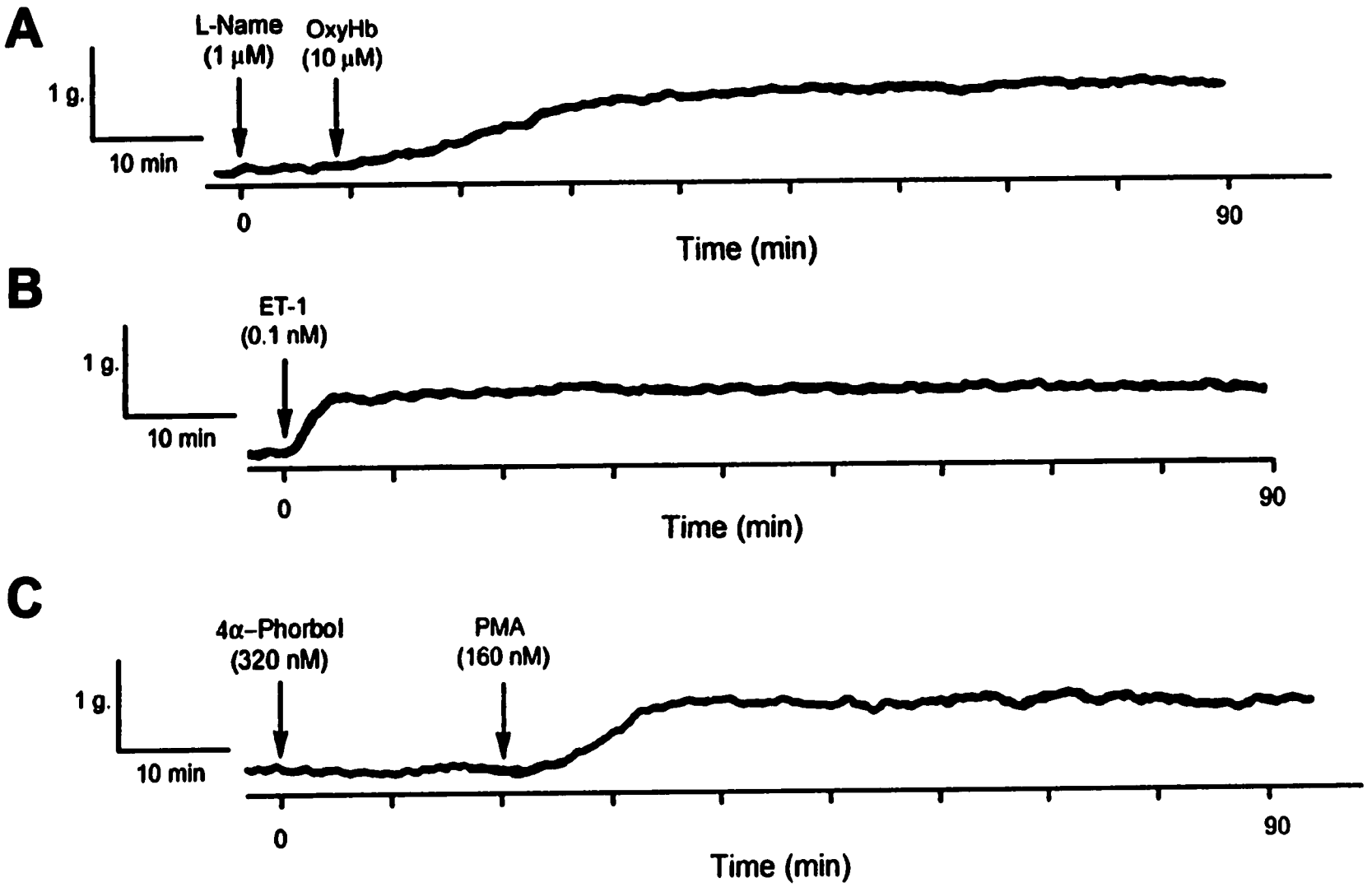

A
L-Name
(1 μM)
OxyHb
(10 μM)
1 g.
10 min
0
90
Time (min)
B
ET-1
(0.1 nM)
1 g.
10 min
0
90
Time (min)
C
4α-Phorbol
(320 nM)
PMA
(160 nM)
1 g.
10 min
0
90
Time (min)

Fig 5. Effects of the ET_A receptor antagonist, BQ-123, on ET-1 induced contraction. Cumulative concentration-effect curves for the vasoconstriction of basilar artery rings elicited by ET-1 in the presence and absence of the selective ET_A receptor antagonist, BQ-123. ET-1 contracted preparations with an EC_{50} of 0.48 nM. Values are expressed as a percentage of the maximum response observed in the absence of antagonist. Data points represent means ± s.e.m. for measurements with four to five ring preparations.

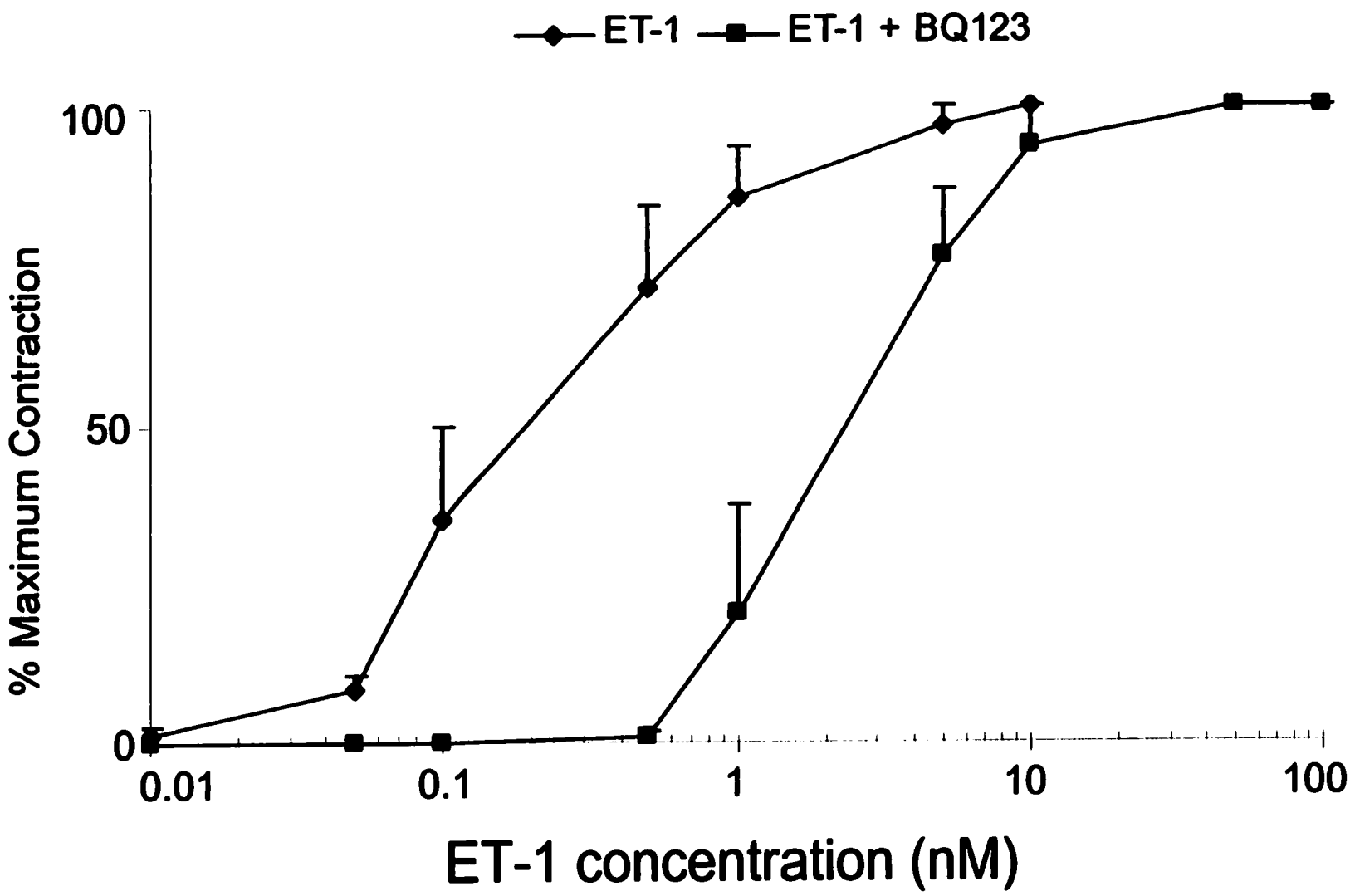

ET-1
ET-1 + BQ123
100
50
0
% Maximum Contraction
0.01
0.1
1
10
100
ET-1 concentration (nM)

Fig 6. Effect of bradykinin on serotonin-induced contraction of basilar arteries. Representative trace of the effects of bradykinin on rings of basilar artery with denuded endothelium. The rings were contracted with 5-hydroxytryptamine (5-HT) (10 μM). Bradykinin produced no relaxation suggesting that the endothelium had been removed from the preparations. The tracing is representative of three independent experiments. Arrows indicate time of administration of 5-HT and bradykinin.

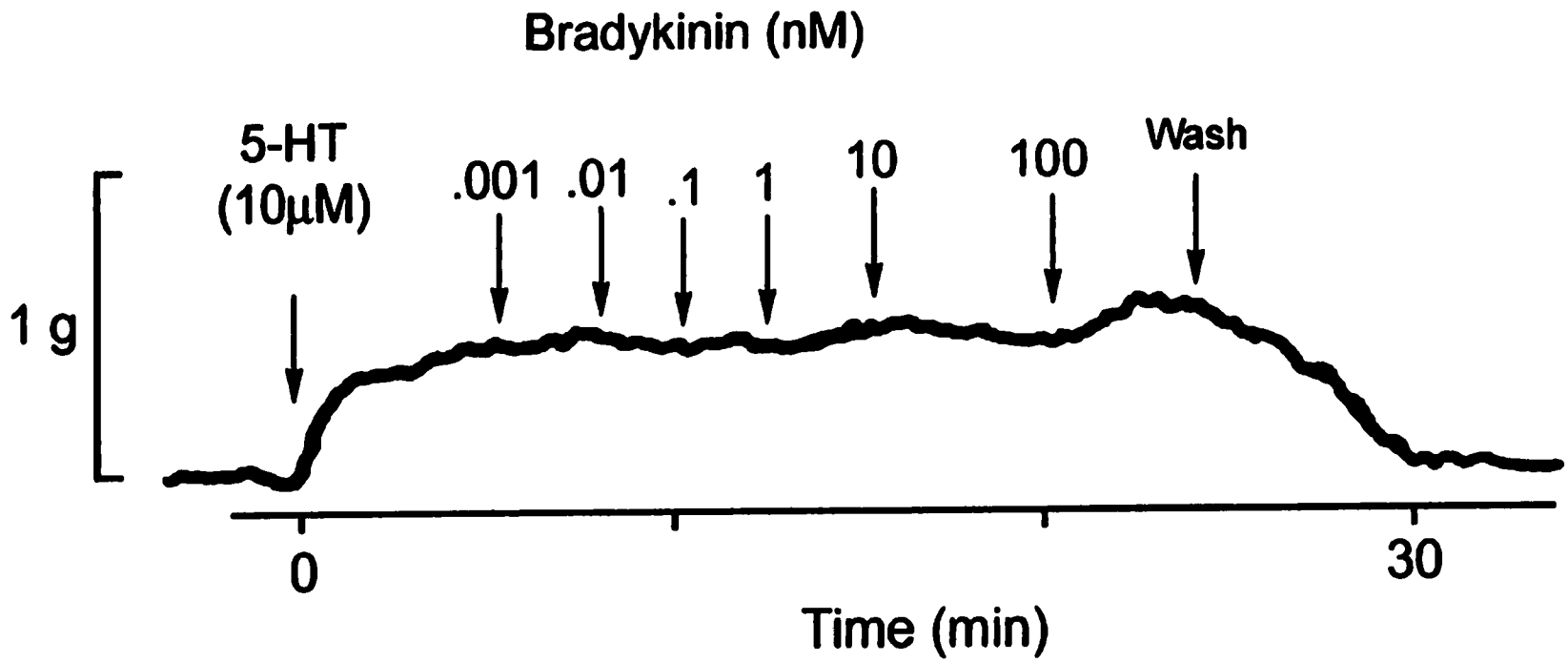

Bradykinin (nM)
5-HT
(10µM)
.001
.01
.1
1
10
100
Wash
1 g
0
30
Time (min)

Fig 7. Representative traces of the effects of the PKC inhibitor, Ro32-0432, on vasoconstriction of basilar artery rings induced by OxyHb (10 μM) (panel A), ET-1 (0.1 nM) (panel B), and PMA (160 nM) (panel C). Ro32-0432 was applied in increased cumulative concentrations (56-360 μM) after tonic responses had developed.

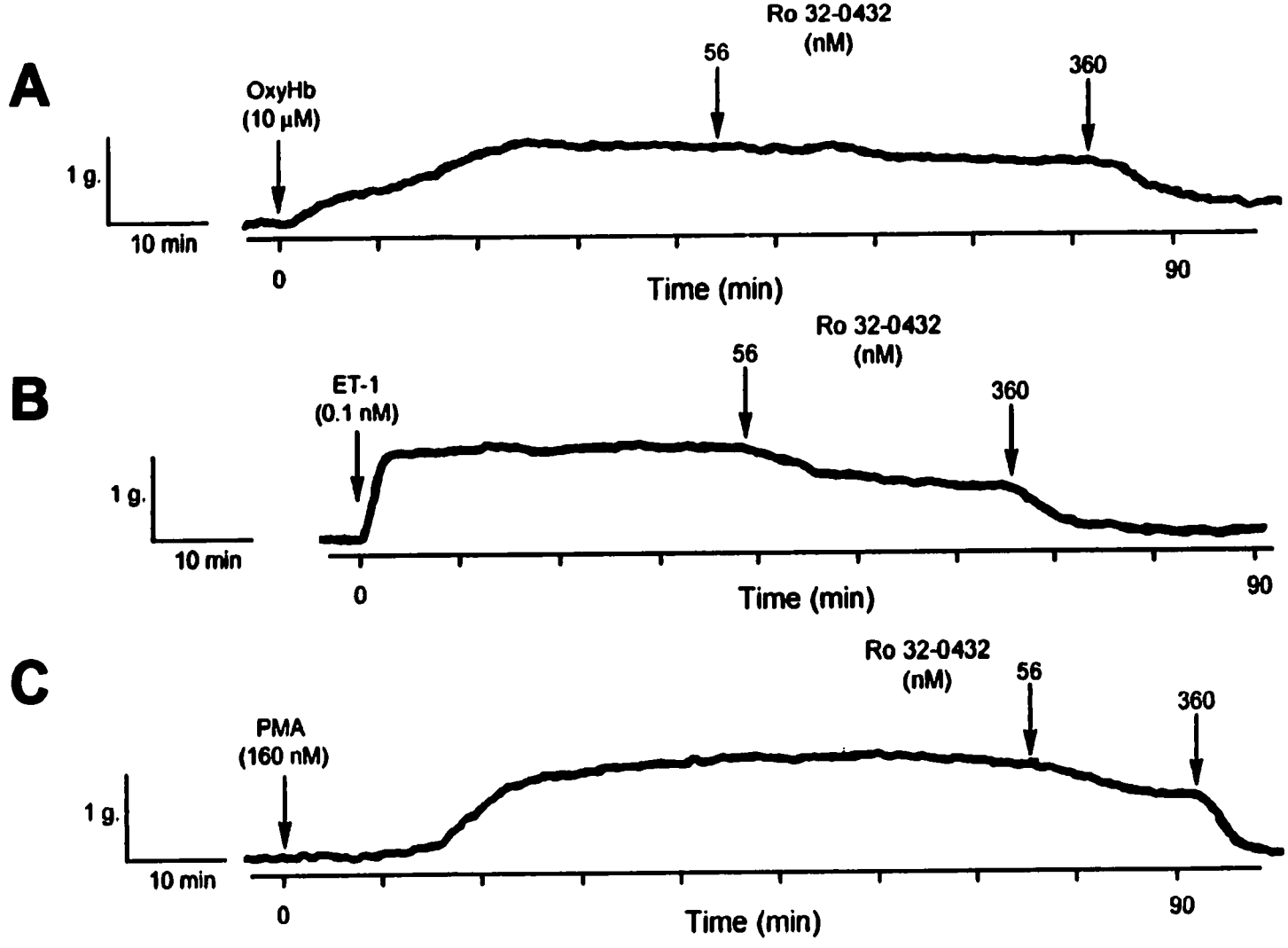

A
OxyHb
(10 µM)
Ro 32-0432
(nM)
56
360
1 g.
10 min
0
90
Time (min)
B
ET-1
(0.1 nM)
Ro 32-0432
(nM)
56
360
1 g.
10 min
0
90
Time (min)
C
PMA
(160 nM)
Ro 32-0432
(nM)
56
360
1 g.
10 min
0
90
Time (min)

Fig 8. Effects of the PKC inhibitor, Ro32-0432, on vasoconstriction induced in rings of basilar artery by OxyHb (10 μM), ET-1 (0.5 nM), and PMA (160 nM). The ring preparations were exposed to Ro32-0432 after tonic contraction to these agents had developed. The responses are expressed as the percentage of maximum tonic tension observed in the absence of inhibitor. Bars represent mean ± s.e.m., with n number indicated below. Asterisks indicate statistical significance, *$p<0.05$, **$p<0.01$, compared to their respective controls.

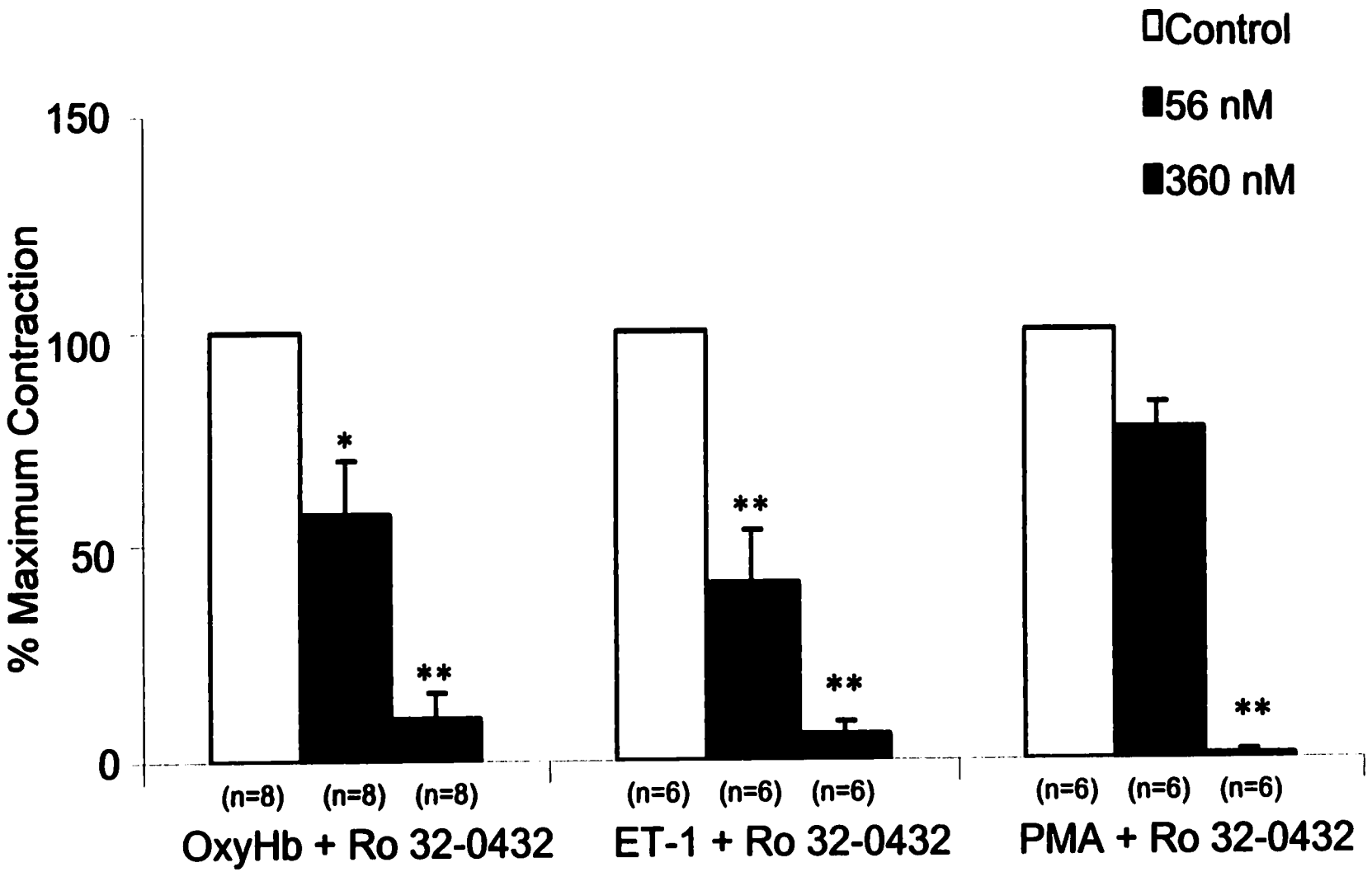

Control
56 nM
360 nM
% Maximum Contraction
150
100
50
0
*
**
**
**
**
(n=8) (n=8) (n=8)
(n=6) (n=6) (n=6)
(n=6) (n=6) (n=6)
OxyHb + Ro 32-0432
ET-1 + Ro 32-0432
PMA + Ro 32-0432

Fig 9. Representative traces of the effects of the PKC inhibitor Gö 6976, on basilar artery ring vasoconstriction induced by OxyHb (10 μM) (panel A), ET-1 (0.1 nM) (panel B), and PMA (160 nM) (panel C). Gö 6976 was applied in increasing cumulative concentrations (5-100 nM) after tonic response had developed. Arrows denote time of administration of indicated compounds.

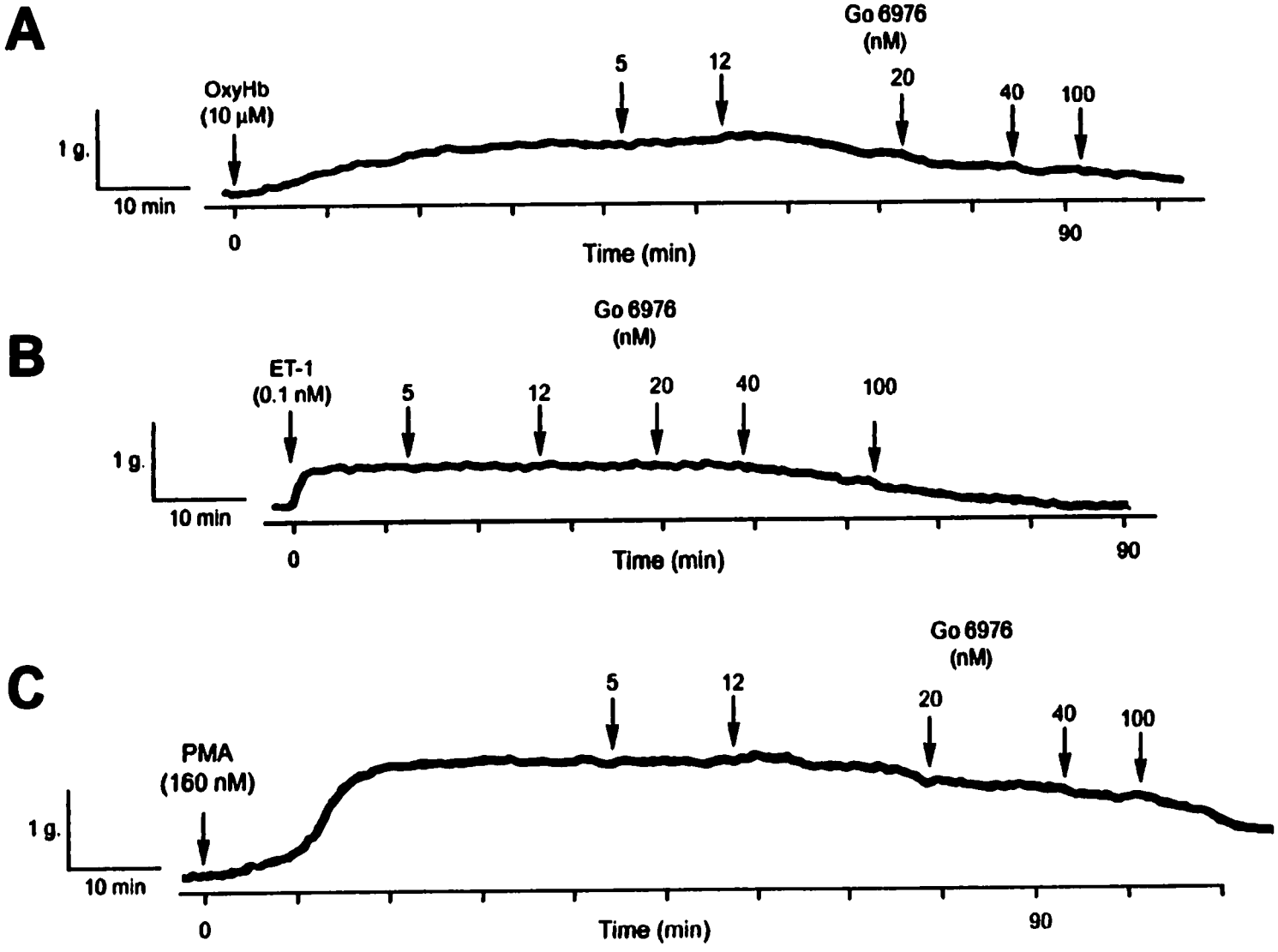

A
Go 6976
(nM)
5
12
20
40
100
OxyHb
(10 µM)
1 g.
10 min
0
90
Time (min)
B
Go 6976
(nM)
ET-1
(0.1 nM)
5
12
20
40
100
1 g.
10 min
0
90
Time (min)
C
Go 6976
(nM)
5
12
20
40
100
PMA
(160 nM)
1 g.
10 min
0
90
Time (min)

Fig 10. PKC activity measurements of cytosolic and membrane fractions of CVSMC exposed to OxyHb (10 μM) for 5 min, 15 min, 60 min, and 24 hours. Activity data is expressed as a percentage of untreated controls (% Control Activity). Bars represent mean ± s.e.m., with n number indicated below. Asterisks indicate statistical significance, **$p<0.01$, compared to their respective control values.

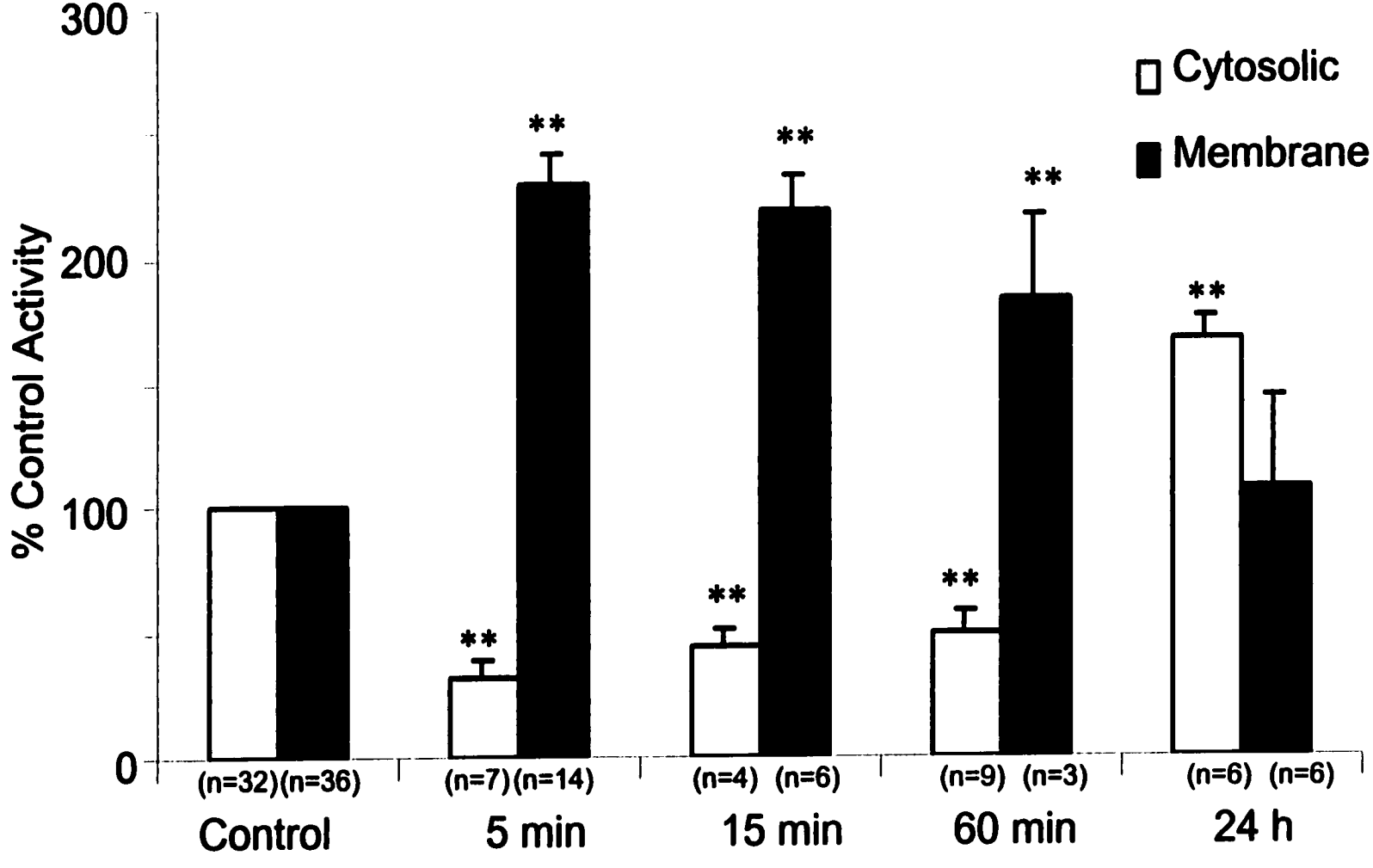

300
200
100
0
% Control Activity
Cytosolic
Membrane
**
**
**
**
**
(n=32)(n=36)
(n=7)(n=14)
(n=4) (n=6)
(n=9) (n=3)
(n=6) (n=6)
Control
5 min
15 min
60 min
24 h

Fig 11. PKC activity measurements of cytosolic and membrane fractions of CVSMC exposed to ET-1 (0.5 nM) for 5 min and 60 min. Activity data is expressed as a percentage of untreated controls (% Control Activity). Bars represent mean ± s.e.m., with n number indicated below. Asterisks indicate statistical significance, **$p<0.01$, compared to their respective control values.

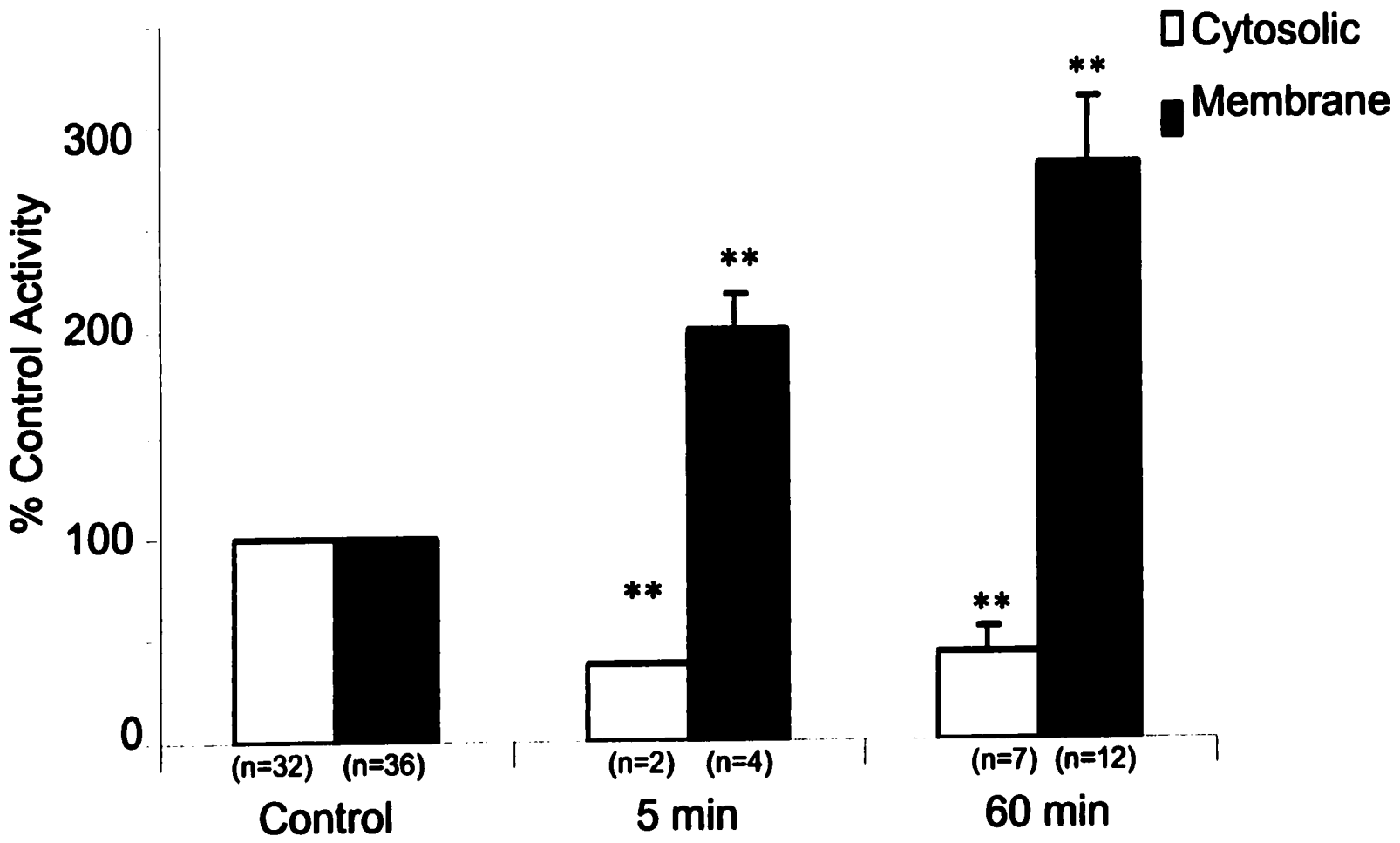

Cytosolic
Membrane
% Control Activity
300
200
100
0
**
**
**
(n=32) (n=36)
(n=2) (n=4)
(n=7) (n=12)
Control
5 min
60 min

Fig 12. PKC activity measurements of cytosolic and membrane fractions of CVSMC exposed to PMA (160 nM) for 5 min, 15 min, and 60 min. Activity data is expressed as a percentage of untreated controls (% Control Activity). Bars represent mean ± s.e.m., with n number indicated below. Asterisks indicate statistical significance, *$p<0.05$, **$p<0.01$, compared to their respective control values.

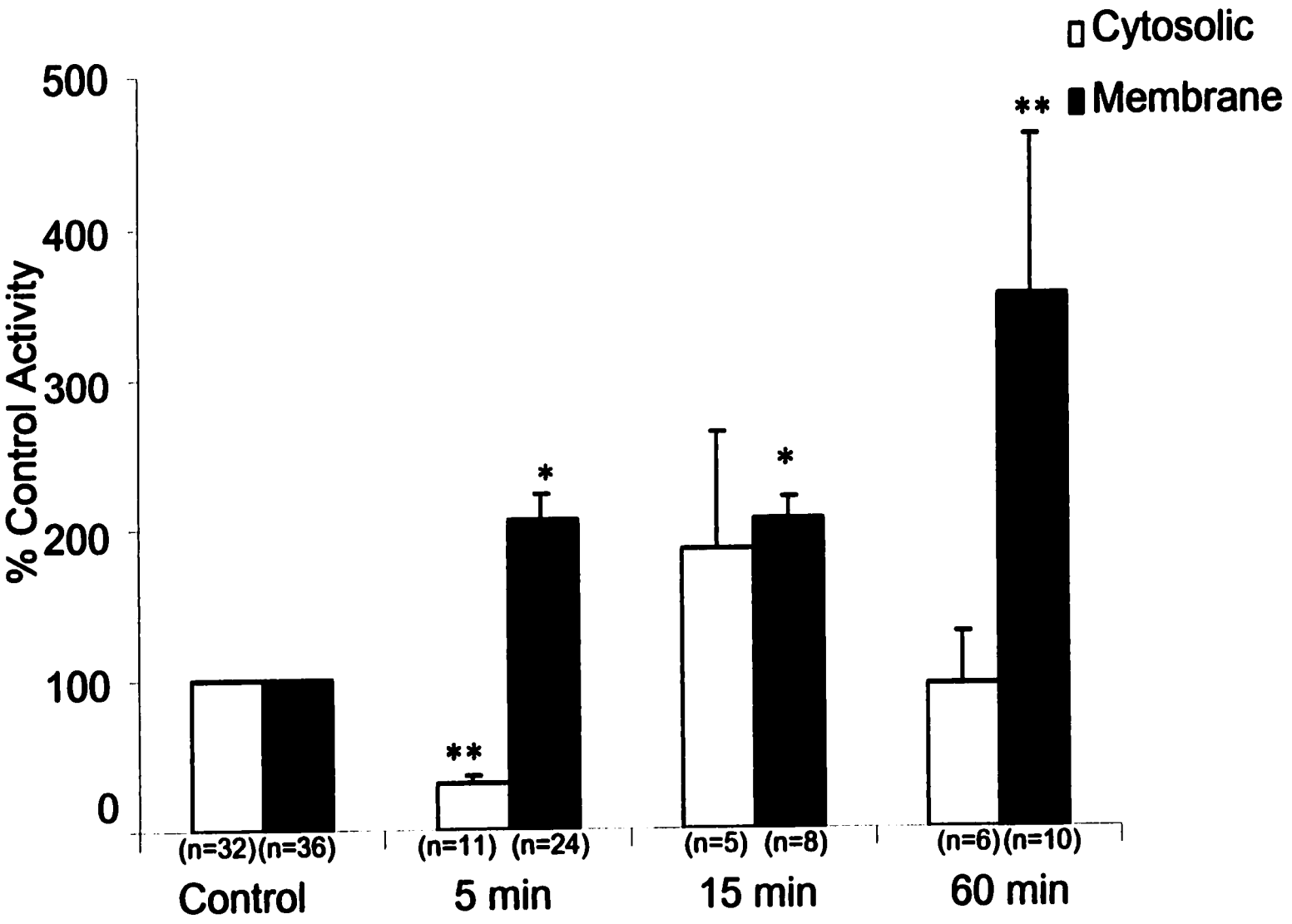

Cytosolic
Membrane
% Control Activity
500
400
300
200
100
0
**
*
*
**
(n=32)(n=36)
(n=11) (n=24)
(n=5) (n=8)
(n=6)(n=10)
Control
5 min
15 min
60 min

Fig 13. Expression profiles of PKC isoforms in cultured cerebrovascular smooth muscle and intact basilar artery. The samples were subjected to SDS-PAGE electrophoresis simultaneously with rat brain homogenate as a positive control. Western blots were then cut and probed with isoform specific antibodies. Expression of the α, δ, ε, and λ isoforms was detected in cultured cells (panel A) while the α, β, ε, λ isoforms were detected in intact arteries (panel B). The molecular weight marker of 77 kDa is shown on the left and corresponds to the molecular weight of PKC isoforms (74-90 kDa).

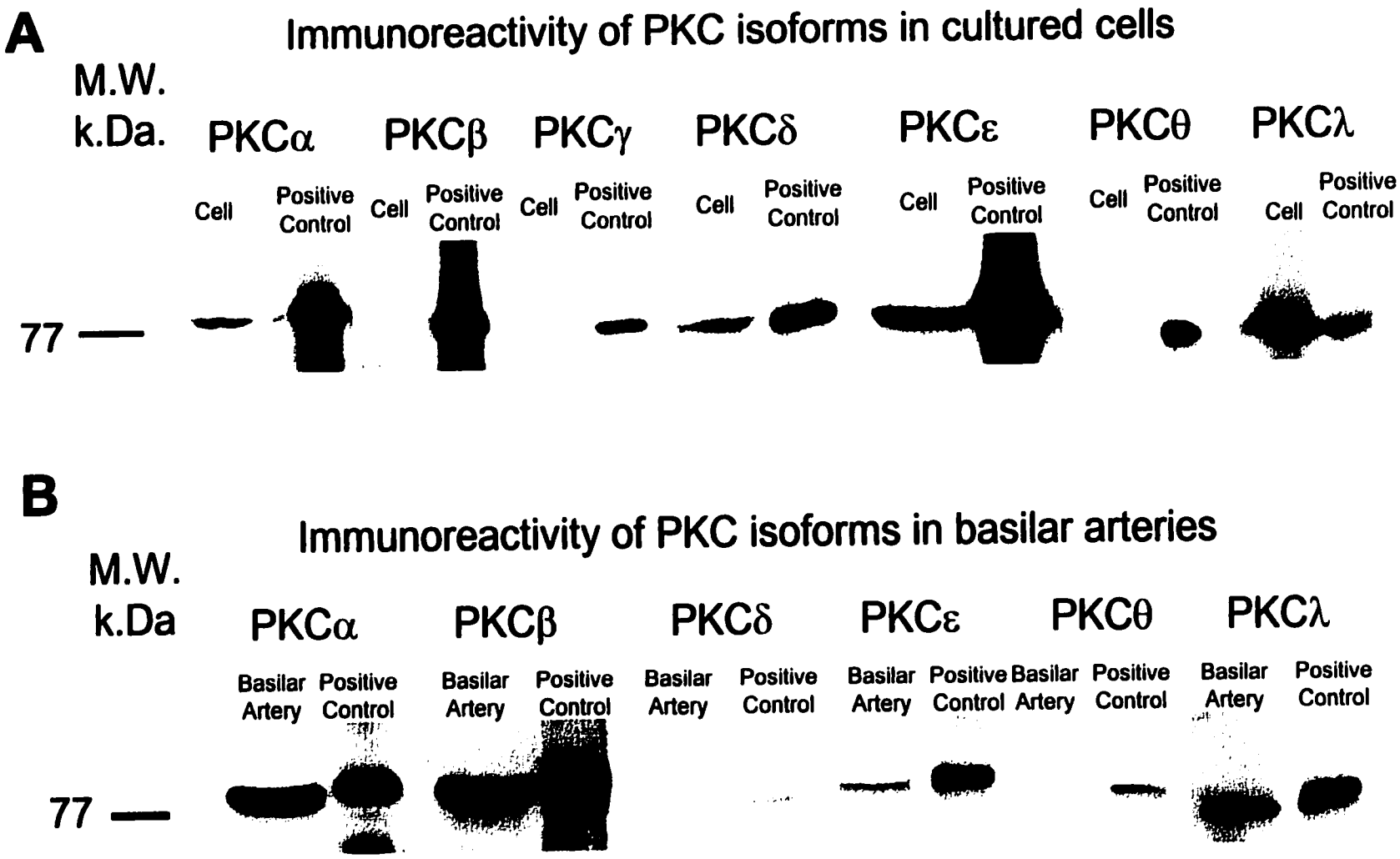

A
Immunoreactivity of PKC isoforms in cultured cells
M.W.
k.Da.
PKCα
PKCβ
PKCγ
PKCδ
PKCε
PKCθ
PKCλ
Cell
Positive Control
77
B
Immunoreactivity of PKC isoforms in basilar arteries
M.W.
k.Da
PKCα
PKCβ
PKCδ
PKCε
PKCθ
PKCλ
Basilar Artery
Positive Control
77

Fig 14. Translocation of PKCα to membrane fractions in CVSMC following 60 min stimulation with Oxyb (10 μM) and PMA (160 nM). Fractionated homogenates were probed with anti-PKCα antibody and a representative western blot is shown in panel A. The position of PKCα is indicated on the right and that of molecular weight marker (kDa) on the left of the blot. The density of the individual bands was measured by densitometry and expressed in arbitrary units, shown in panel B. Each bar represents mean ± s.e.m. of 4 different blots. Asterisks indicate statistical significance, *$p<0.05$, **$p<0.01$, compared to their respective control values.

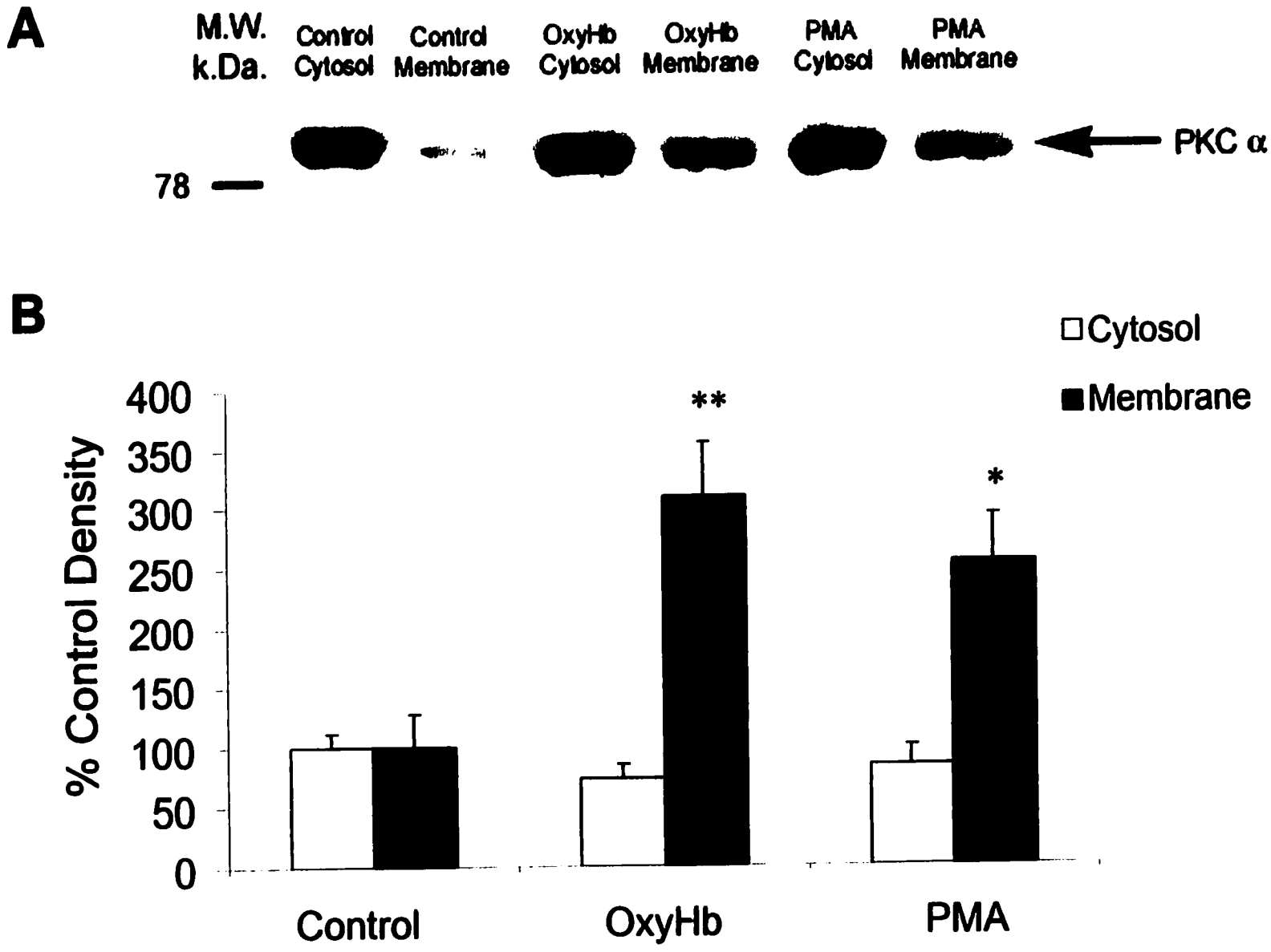

A
M.W.
k.Da.
Control Cytosol
Control Membrane
OxyHb Cytosol
OxyHb Membrane
PMA Cytosol
PMA Membrane
78
PKC α
B
Cytosol
Membrane
% Control Density
400
350
300
250
200
150
100
50
0
**
*
Control
OxyHb
PMA

Fig 15. Translocation of PKCε to membrane fractions in CVSMC following 60 min stimulation with Oxyb (10 μM) and PMA (160 nM). Fractionated homogenates were probed with anti-PKCε antibody and a representative western blot is shown in panel A. The position of PKCε is indicated on the right and that of molecular weight marker (kDa) on the left of the blot. The density of the individual bands was measured by densitometry and expressed in arbitrary units, shown in panel B. Each bar represents mean ± s.e.m. of 4 different blots. Asterisks indicate statistical significance, *$p<0.05$, compared to their respective control values.

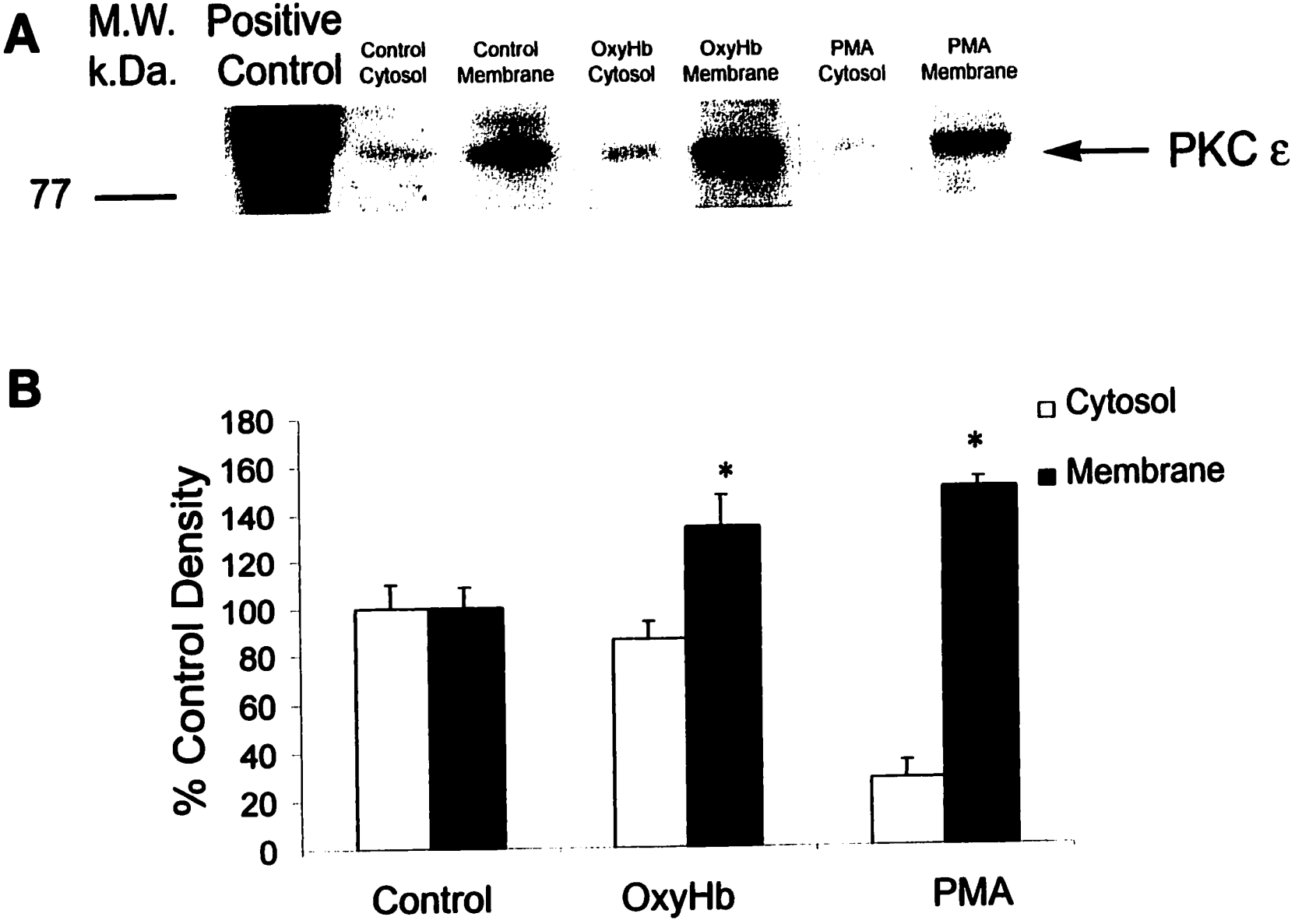

A
M.W.
k.Da.
Positive
Control
Control
Cytosol
Control
Membrane
OxyHb
Cytosol
OxyHb
Membrane
PMA
Cytosol
PMA
Membrane
77
PKC ε
B
% Control Density
180
160
140
120
100
80
60
40
20
0
Cytosol
Membrane
*
*
Control
OxyHb
PMA

Fig 16. Effect of tamoxifen on OxyHb, ET-1, and PMA induced contraction. Representative traces of the relaxant effects of cumulative concentrations of tamoxifen (0.1-5.0 μM) against basilar artery ring contraction induced by OxyHb (10 μM) (panel A), ET-1 (50 nM) (panel B), and PMA (160 nM) (panel C). Tamoxifen was added during maximum tonic contraction. Arrows denote time of administration of indicated compounds.

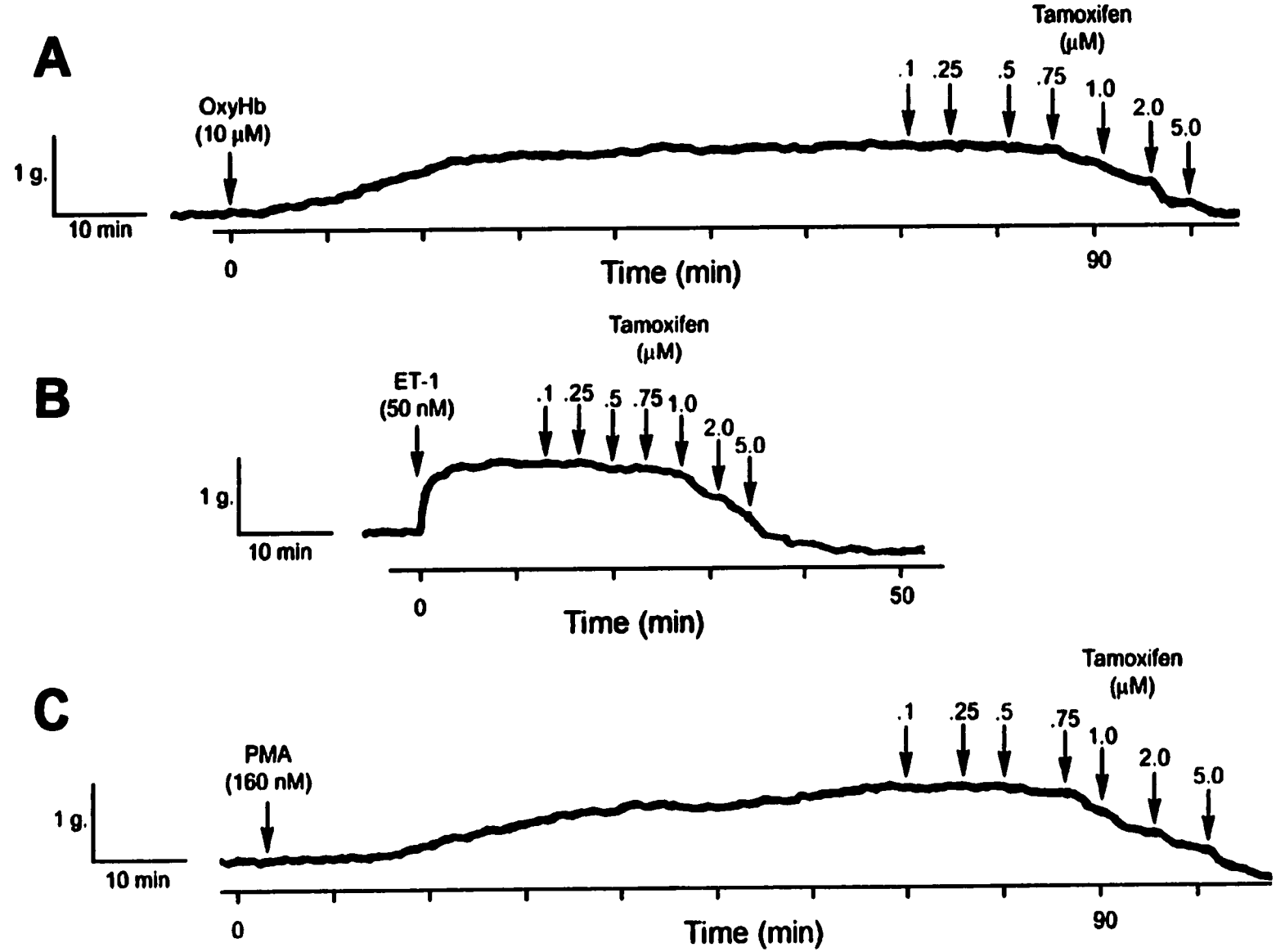

A
Tamoxifen
(μM)
.1 .25 .5 .75 1.0 2.0 5.0
OxyHb
(10 μM)
1 g.
10 min
0
Time (min)
90
B
Tamoxifen
(μM)
ET-1
(50 nM)
.1 .25 .5 .75 1.0 2.0 5.0
1 g.
10 min
0
Time (min)
50
C
Tamoxifen
(μM)
.1 .25 .5 .75 1.0 2.0 5.0
PMA
(160 nM)
1 g.
10 min
0
Time (min)
90

Fig 17. Cumulative concentration-response curves for tamoxifen induced relaxation of basilar artery preparations. Arterial rings denuded of endothelium were pre-contracted with OxyHb (10mM), ET-1 (50 nM), KCl (60 mM), or PMA (160 nM). Data are expressed as the mean ± s.e.m. for experiments conducted with seven or more ring preparations from five animals.

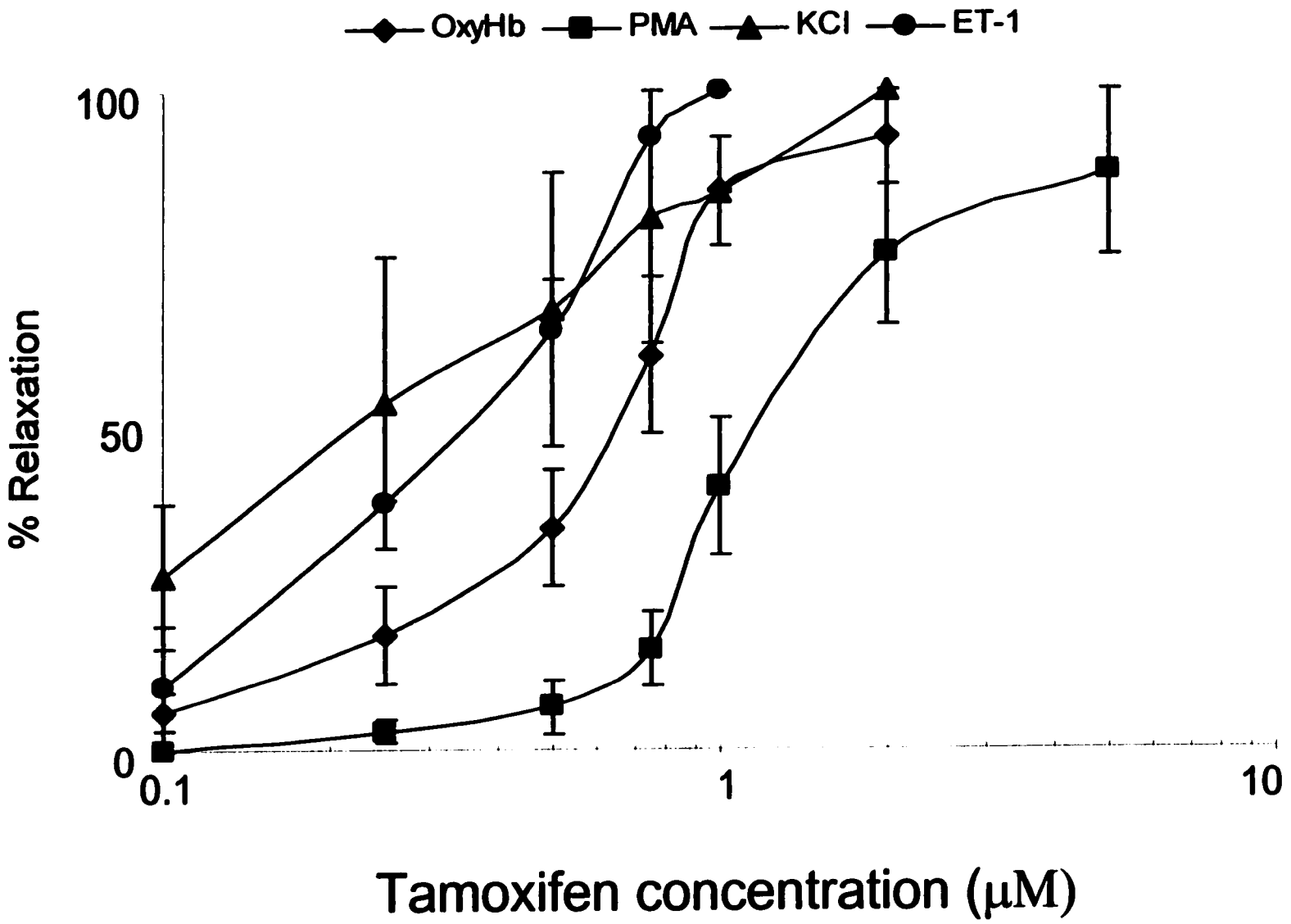

OxyHb
PMA
KCl
ET-1
100
50
0
% Relaxation
0.1
1
10
Tamoxifen concentration (μM)

Fig 18. Effects of tamoxifen on protein kinase C activity in the membrane fractions from cerebrovascular smooth muscle cells exposed to OxyHb (10 μM) and PMA (160 nM) for 5 min. Tamoxifen (7 μM) was administered 30 min before application of OxyHb or PMA. Activity data is expressed as a percentage of untreated controls (% Control Activity). Bars represent mean ± s.e.m. Asterisks indicate statistical significance, *$p<0.05$, **$p<0.01$ vs control; ##$p<.01$ vs OxyHb or PMA alone.

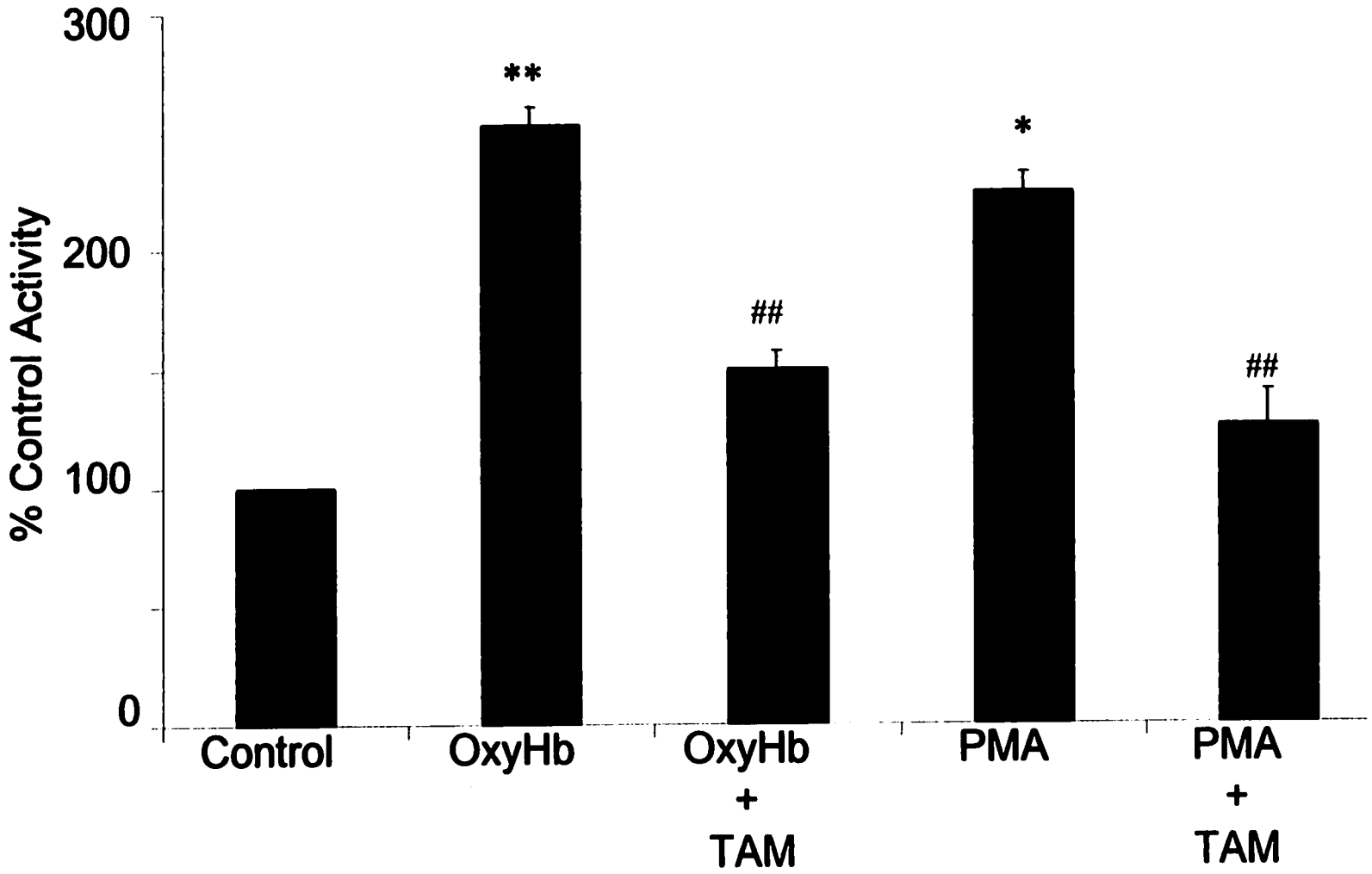

300
200
100
0
% Control Activity
**
*
##
##
Control
OxyHb
OxyHb + TAM
PMA
PMA + TAM

Fig 19. Representative traces of the responses of rings of basilar arteries contracted with ET-1 (0.5 nM) (panel A) and PMA (160 nM) (panel B) to gentamicin (0.01-5.0 mM). Gentamicin was added in cumulative increasing concentrations after a tonic response had developed. Arrows denote time of administration of indicated compounds.

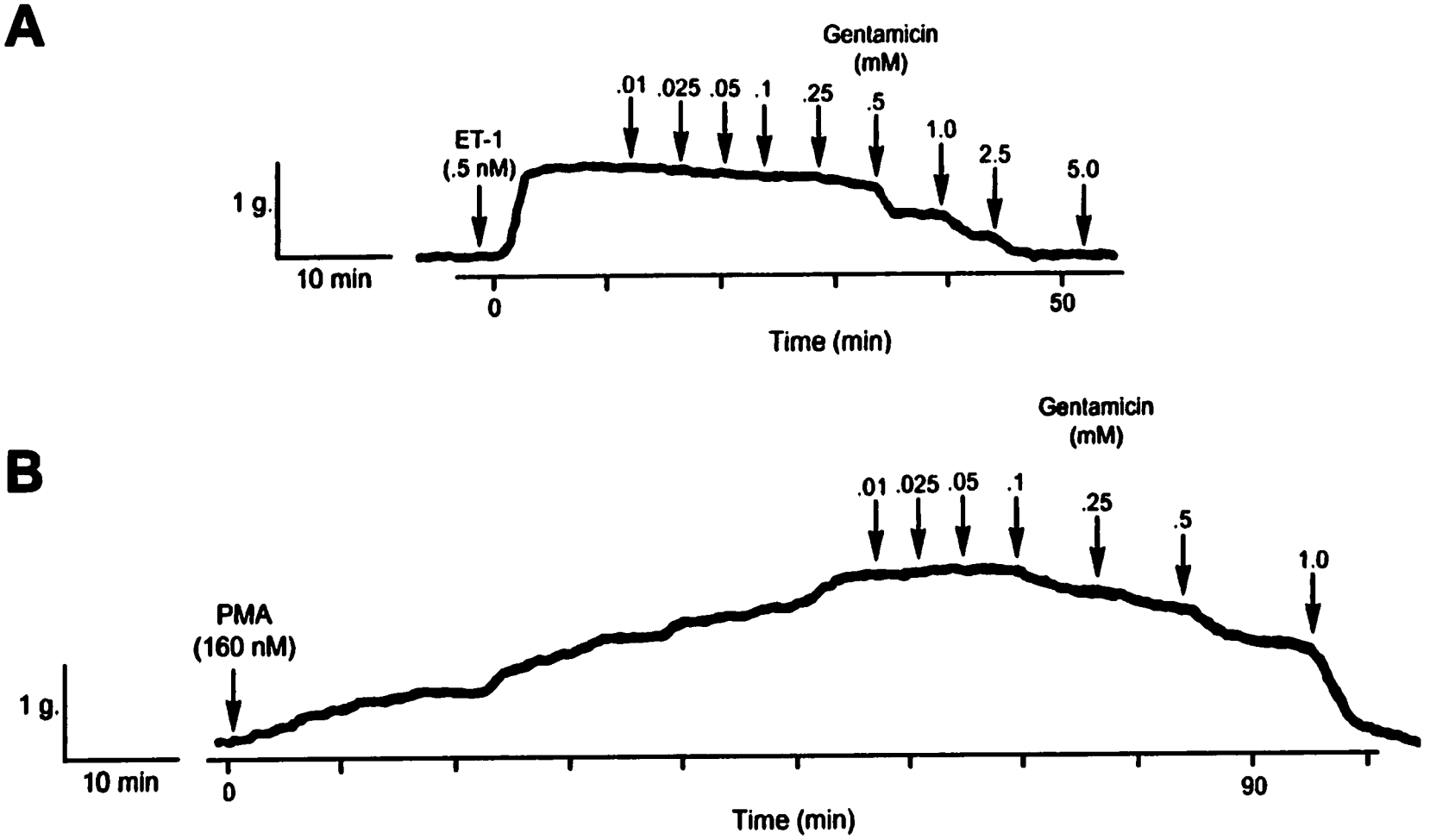

A
Gentamicin
(mM)
.01 .025 .05 .1 .25 .5 1.0 2.5 5.0
ET-1
(.5 nM)
1 g.
10 min
0
50
Time (min)
B
Gentamicin
(mM)
.01 .025 .05 .1 .25 .5 1.0
PMA
(160 nM)
1 g.
10 min
0
90
Time (min)

Fig 20. Relaxant effect of gentamicin and Ro32-0432 on PMA-induced contraction. Representative traces of the vasoconstrictor responses induced by PMA (160 nM) in calcium free medium. Gentamicin (panel A) and Ro32-0432 (panel B) were administered to the preparations in which maximum tonic contraction to PMA had developed. Each trace, which illustrates the response of an individual ring preparation, is representative of four independent experiments conducted with ring preparations from four different animals.

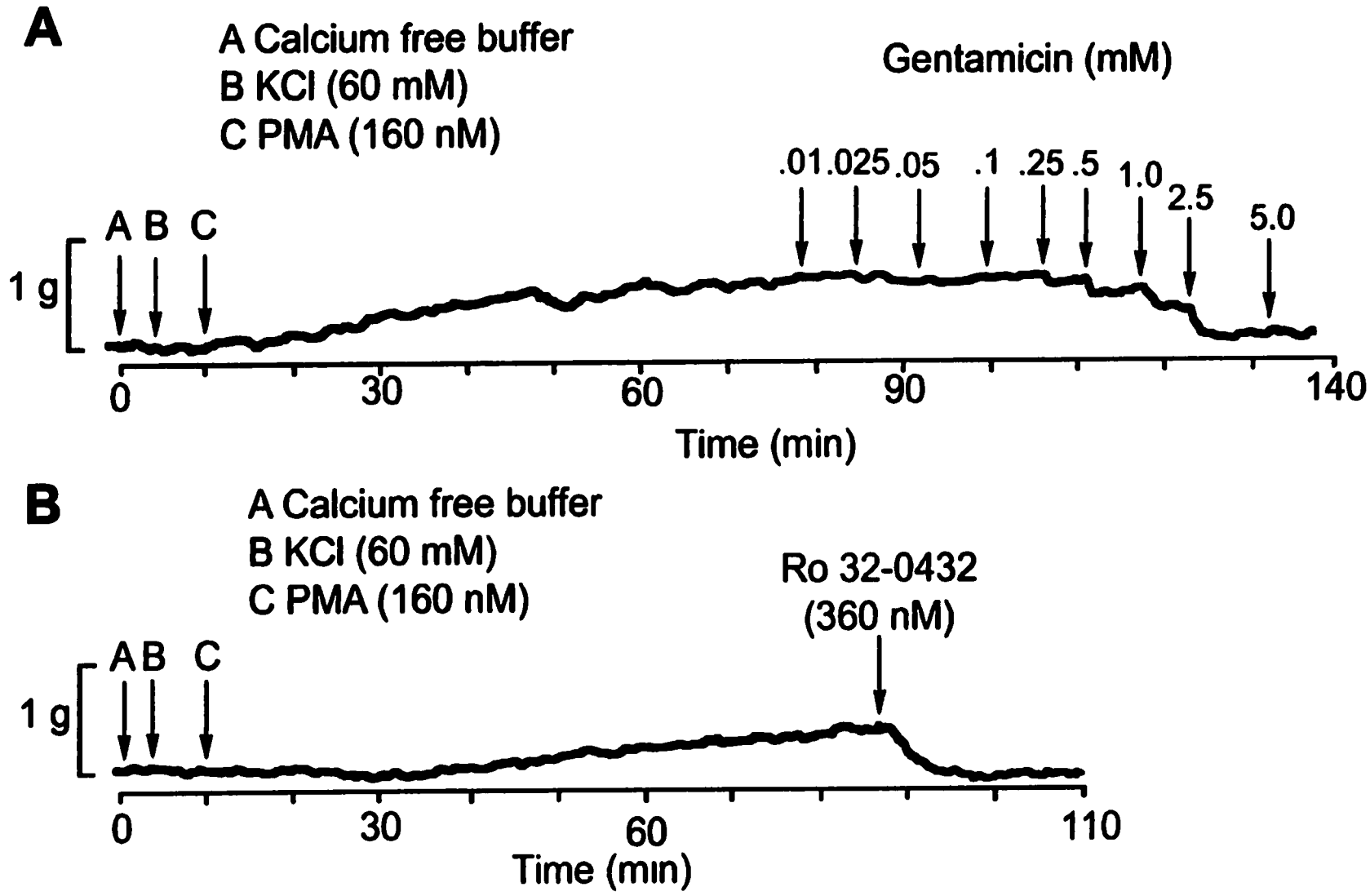
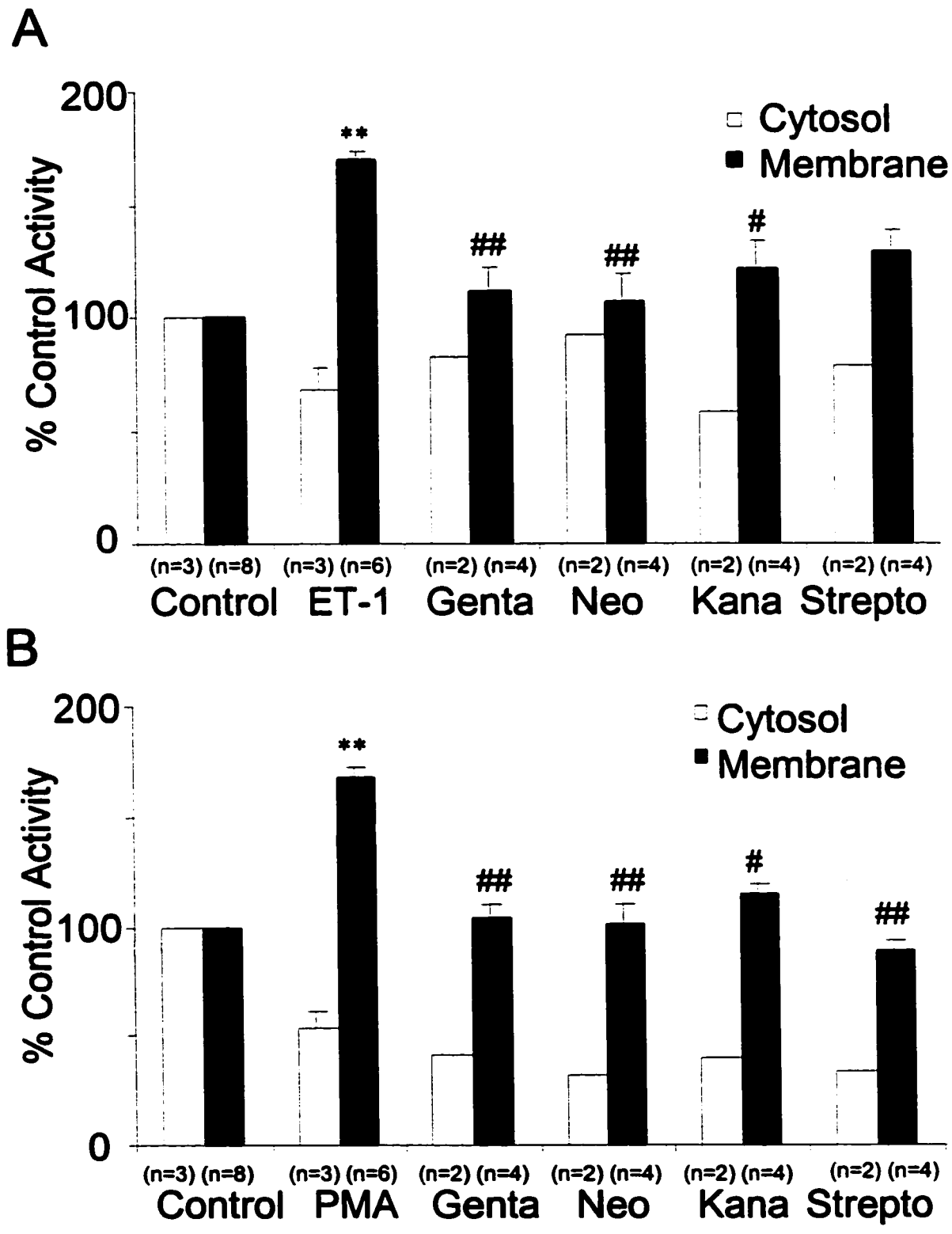

A
A Calcium free buffer
B KCl (60 mM)
C PMA (160 nM)
Gentamicin (mM)
.01 .025 .05 .1 .25 .5 1.0 2.5 5.0
A B C
1 g
0 30 60 90 140
Time (min)
B
A Calcium free buffer
B KCl (60 mM)
C PMA (160 nM)
Ro 32-0432
(360 nM)
A B C
1 g
0 30 60 110
Time (min)

Fig 21. Effects of the aminoglycoside antibiotics on PKC activity in CVSMC. PKC activity was determined in the soluble and particulate fractions of cells exposed to ET-1 (0.5 nM) (panel A) or PMA (160 nM) (panel B) for 60 min. The aminoglycosides, gentamicin (genta) (1.5 mM), neomycin (neo) (1.6 mM), kanamycin (kana) 4.5 mM), and streptomycin (strepto) (1.4 mM), were administered 30 min after drug application, in concentrations corresponding to EC_{50} values obtained from contractility experiments.Each bar represents mean ± s.e.m. of 6 experiments. Asterisks indicate statistical significance, **$p<0.01$ vs control; #$p<0.01$ vs stimulation with ET-1or PMA alone; ##$p<0.001$ vs stimulation with ET-1 or PMA alone.


A
200
100
0
% Control Activity
Cytosol
Membrane
**
##
##
#
(n=3) (n=8)
(n=3) (n=6)
(n=2) (n=4)
(n=2) (n=4)
(n=2) (n=4)
(n=2) (n=4)
Control
ET-1
Genta
Neo
Kana
Strepto
B
200
100
0
% Control Activity
Cytosol
Membrane
**
##
##
#
##
(n=3) (n=8)
(n=3) (n=6)
(n=2) (n=4)
(n=2) (n=4)
(n=2) (n=4)
(n=2) (n=4)
Control
PMA
Genta
Neo
Kana
Strepto

CHAPTER 4:

4.0 DISCUSSION

The pathogenesis of cerebral vasospasm following subarachnoid hemorrhage is believed to be multifactorial (Cook and Vollrath, 1995). The compounds OxyHb and ET-1 are the primary vasoconstrictors implicated in the pathogenesis of vasopasm and have been hypothesized to act by a variety of mechanisms, including activation of PKC (Cook and Vollrath, 1995). The involvement of PKC in vasospasm has been studied in animal models of the disease and have shown that during vasospasm there is an increase in membrane-bound PKC, a strong indication of activation (Megyesi *et al.*, 2000 ; Sako *et al.*, 1993). In addition, intracellular levels of diacylglycerol (DAG) have been found to be elevated during vasospasm which would be consistent with a prolonged activation of PKC (Matsui *et al.*, 1991.). Furthermore, the PKC inhibitors H-7 and staurosporine induce significant dilation of spastic basilar arteries (Matsui *et al.*, 1991), indicating that vasospasm may be due to the activation of the PKC-dependent contractile system. However, in these earlier studies the involvement of PKC was investigated days following induction of vasospasm, and gave no indication as to whether PKC may be involved in the initiation of prolonged vascular contraction due to OxyHb and ET-1. Thus, the present studies investigated the role of PKC in cerebral artery contraction induced by OxyHb and ET-1, the primary agents implicated in the pathogenesis of vasospasm. An additional objective of these studies was to examine tamoxifen and aminoglycoside-induced relaxation of vascular contraction. These compounds have been reported to be effective inhibitors of some of the key processes which are believed to be involved in the development of vasospasm. Thus the efficacy and mechanism of action of tamoxifen and the aminoglycoside antibiotics were assessed in order to determine their

potential as pharmacotherapy for cerebral vasospasm following subarachnoid hemorrhage.

4.1 ROLE OF PKC IN CEREBROVASCULAR CONTRACTION

4.1.1 EFFECTS OF OXYHB, ET-1, AND PMA ON VASCULAR CONTRACTILITY

It is now widely accepted that cerebral vasospasm represents a contraction of vascular smooth muscle (Kassel et al., 1985). The present studies demonstrated that OxyHb produced a slowly developing sustained contraction of isolated cerebral blood vessels. Cerebrovascular contractile responses to ET-1 occurred more rapidly but were also characterized by a prolonged contraction. These observations confirm the contractile ability of these agents in isolated cerebrovascular preparations as well as support their potential role in the pathogenesis of vasospasm.

Role of calcium in vasoconstriction

The vascular contraction produced in response to these agents is a result of the activation of complex intracellular signaling pathways. The contractile responses mediated by ET-1 were inhibited with the ET_A receptor antagonist, BQ-123, indicating that the contractile action of this peptide occurs via the ET_A receptor subtype coupled to PLC. Activation of this pathway is known to produce an increase in intracellular calcium, by multiple mechanisms, which acutely stimulates vascular contraction. In addition, exposure of smooth muscle cells to OxyHb is also associated with an elevation of intracellular calcium which has been proposed to be due to either calcium influx (Steele *et al.*, 1991) or the liberation of Ca^{2+} from the intracellular stores due to activation

of PLC and the subsequent generation of inositol trisphosphate (Vollrath *et al.*, 1990.). These observations imply that the prolonged smooth muscle contraction is due to the activation of the Ca^{2+}-CaM dependent contractile pathway. However, the ratio of phosphorylated to unphosphorylated myosin is unchanged in spastic arteries compared to that in normal arteries (Sun *et al.*, 1998). In addition, neither calcium channel antagonists (Varsos *et al.*, 1983) nor calmodulin inhibitors (Peterson *et al.*, 1989) prevent the occurrence of cerebral vasospasm or dilate spastic arteries (Nosko *et al.*, 1985). Therefore, activation of the Ca^{2+}-CaM dependent contractile pathway alone cannot explain the prolonged vascular contraction characteristic of vasospasm. The intracellular pathways stimulated by OxyHb and ET-1 are also known to activate PKC and there is evidence to suggest that this may be a substantial component of their contractile mechanisms of action (Asano and Matsui, 1999).

Role of PKC in vasoconstriction

Previous observations strongly suggested a central role for PKC-mediated contraction of cerebral arteries following SAH, and the present studies sought to further explore this possibility with the PKC antagonists, Ro32-0432 and Gö 6976. Ro32-0432 is a member of the bisindolylmaleimide family of PKC inhibitors which display a tenfold selectivity for classical isoforms over the novel. These inhibitors compete with ATP binding sites in the catalytic subunit and in this way they are though to inhibit PKC activity (Wilkinson *et al.*, 1993). The PKC inhibitor Gö 6976 is a derivative of the indolocarbazole, staurosporine, and inhibits the classic calcium-dependent isozymes at nanomolar concentrations while having no effect on the kinase activity of the novel

calcium independent subtypes. Gö 6976 inhibits enzymatic activity of PKC by competing with ATP for binding to the enzyme (Martiny-Baron *et al.*, 1993). In these studies we have shown that prolonged vascular contraction stimulated by OxyHb and ET-1 was attenuated with both Ro32-0432 and Gö 6976, at 60 min of contraction, confirming the involvement of PKC in the maintenance of contractile tone and suggesting that PKC plays a central role in the sustained contractile response to these agents. The calcium sensitive classical types of PKC appear to be the primary isoforms responsible for the prolonged vascular contractile response to OxyHb and ET-1. While the studies with Ro32-0432 and Gö 6976 also demonstrate the novel, calcium independent PKC isoform ε, is also involved in the initiation and maintenance of cerebrovascular smooth muscle contraction its activation appears to be less important in the contractile process initiated by OxyHb or ET-1.

Tumor promoting phorbol esters were employed to investigate the effects of selective PKC activation of smooth muscle contractility. Phorbol esters bind to specific protein cysteine residues present in the regulatory subunit of PKC and selectively activate this enzyme (Gschwendt *et al.*, 1991). When exposed to isolated basilar ring preparations the phorbol ester, PMA, produced a slowly developing sustained constriction that could not be reversed by removal of the compound. The observation that phorbol ester-induced contraction is not easily relaxed, even when the agent is removed, further illustrates the potency of PKC-mediated contraction and is highly consistent with the persistent vascular contraction observed during vasospasm (Macdonald, 1995).

Involvement of the actin binding proteins in vascular contraction

At present the exact nature of PKC mediated vascular contraction is not clear. However, it has been proposed that the contractile effect mediated by this enzyme may be the result of actin binding protein phosphorylation (Horowitz *et al.*, 1996). The actin-binding protein, calponin (CaP), is a primary candidate for PKC mediated contraction. When bound to actin, CaP decreases actin capacity to activate myosin ATPase (Miki *et al.*, 1992) and therefore inhibits contraction. It has been suggested that PKC-dependent phosphorylation of CaP attenuates its actin binding capacity, thereby relieving CaP induced inhibition of contraction. When applied exogenously to contracted permeabilized smooth muscle, CaP inhibits contraction. Yet when phosphorylated CaP was applied to the same preparation there was no effect on contraction (Horowitz *et al.*, 1996).. However, additional evidence suggests that an alternative pathway may also be responsible for PKC mediated-contraction.

Phorbol esters, in addition to being powerful initiators of smooth muscle contraction, stimulate the phosphorylation of protein tyrosine residues which results in the activation of subsequent signaling cascades. Although protein tyrosine phosphorylation is known to be a mitogenic signal initiating cellular proliferation and hypertrophy, it is now believed that this process is also involved in smooth muscle contraction (Takahashi and Berk, 1998). The observation that phorbol esters initiate activation of these cascades has led to the hypothesis that PKC may induce vascular smooth muscle contraction through the activation of tyrosine kinase signaling cascades. PKC has been shown to initiate indirectly tyrosine phosphorylation leading to the activation of mitogen activated protein kinases (MAPK), which have been implicated in

smooth muscle contraction and cell proliferation (Khalil and Morgan, 1993). PKC activates cRaf-1, a serine/threonine kinase, and triggers a cascade of phosphorylation events leading to the phosphorylation and activation of MAPK (Marquardt *et al.*, 1994). The mechanism by which MAPK mediates contraction of vascular smooth muscle is not well understood although it has been proposed to act via the actin binding protein, caldesmon. Activated MAPK has been found to co-localize with caldesmon, a thin filament-associated protein similar to CaP (Takahashi and Berk, 1998). Caldesmon is able to bind both actin and myosin, and when bound it limits contraction by inhibiting myosin ATPase activity (Hemric and Chalovich, 1988). Phosphorylation of caldesmon reverses its ability to bind actin and therefore to inhibit smooth muscle contraction. Further examination has revealed that activated MAPK phosphorylates caldesmon *in vivo* (Adam and Hathaway, 1993). Thus MAPK may be an important mediator of vascular smooth muscle contraction and, thus, cerebral vasospasm. Evidence from our laboratory has demonstrated that following exposure of cultured cells to OxyHb, a 42 kDa protein corresponding to MAPK becomes tyrosine phosphorylated, a hallmark of its activation. In addition, the tyrosine kinase inhibitors PD98059 and genistein significantly relaxed OxyHb induced contraction of canine basilar arteries (Vollrath *et al.*, 1998). Consistent with the possibility of MAPK activation spastic arteries develop intimal proliferation, fibrosis, and hypertrophy (Tanabe *et al.*, 1978) which suggests MAPK involvement in this process. This evidence suggests that protein tyrosine phosphorylation is involved in sustained vasoconstriction due to OxyHb and may therefore play a role in the pathogenesis of cerebral vasospasm and subsequent cerebral ischemia.

Inhibition of nitric oxide-mediated vasorelaxation

The observation that the haem moiety of OxyHb binds and inactivates nitric oxide, a diffusible vasodilator, has led to the hypothesis that OxyHb-mediated vasoconstriction is due, at least in part, to the inhibition of nitric oxide dependent relaxation (Hongo et al., 1988.). However, we have shown that endothelium-denuded arteries, in which nitric oxide-dependent relaxation is blocked, were responsive to stimulation with OxyHb suggesting an endothelium-independent mechanism of action.

4.1.2 Effects of OxyHb, ET-1, and PMA on PKC activity

The critical role that PKC plays in OxyHb and ET-1-stimulated sustained contraction was further supported by direct studies of PKC activity. The process of PKC activation is associated with a translocation from the cytosol to the plasma membrane where it binds PS and DAG, specific cofactors of activation (Newton, 1997). This feature of PKC makes it possible to determine the extent of cellular enzyme activation by examining the amount of PKC associated with the membrane. We have demonstrated that OxyHb and ET-1 are potent stimulators of membrane associated PKC activity at 60 min, a time corresponding to maximum tonic contraction, which further supports a role for this enzyme in sustained vasconstriction due to these agents. However, a 24 hour exposure of cultured cells to OxyHb, did not result in a significant increase in membrane associated PKC activity. This observation suggests that OxyHb at very long exposure times is unable to maintain PKC activation which is consistent with some reports which failed to show PKC translocation at very long times following induction of vasospasm in basilar arteries (Sako *et al.*, 1993). It has been suggested that the apparent lack of active

PKC following long term exposure to OxyHb, as determined by measurement of membrane associated PKC, may be due to calpain mediated proteolysis (Minami *at al.*, 1992). The calcium-dependent protease, calpain, has been shown to cleave purified PKC in the hinge region between the regulatory and catalytic subunit (Minami *at al.*, 1992). It has been hypothesized that this cleavage will remove the negative enzyme regulation and yield constitutively active PKC (Minami *at al.*, 1992). With this scheme PKC would no longer have to be associated with the membrane in order to be active. Thus, the PKC in the cytosol, which previously was presumed to be inactive, may, in fact, be fully active and therefore capable of initiating vascular contraction. However, PKC cleaved in this manner may only be active for a limited period of time as calpain will eventually completely degrade the enzyme rendering it inactive. This hypothesis would explain the observations that OxyHb fails to stimulate PKC activation for prolonged periods. Alternatively, it is recognized that PKC is a component of a large signaling cascade which results in that activation of many pathways including that of the MAPKs (Kalil *et al.*, 1993). As previously discussed, PKC may activate MAPK through the Raf-1 signaling pathway which may in turn exert contractile effect on vascular smooth muscle. Therefore, it was suggested that PKC may be involved in the initiation and early maintenance of contraction during vasospasm and may activate additional pathways which maintain a powerful contractile tone in the absence of PKC activity. It is likely that during vasospasm both calpain-induced cleavage of PKC and PKC-activated transduction cascades are factors contributing to sustained contraction and more research must be performed to in order to clarify individual contributions of these processes. Regardless of which pathway may be predominant, it is important to note that PKC

activation/activity plays a key role in the development and maintenance of smooth muscle contraction associated with vasospasm.

4.1.3 Effects of OxyHb, ET-1, and PMA on PKC translocation

At present the evidence in favor of a role for PKC in vasospasm is substantial. Yet, PKC is a family of isozymes which, in many studies, is treated as single entity. This simplistic viewpoint does not reflect the complexities of PKC activation nor contribute to our understanding of the role that particular isoforms of PKC may play in the development of cerebral vasospasm. Thus, we have examined the expression of many PKC isoforms in intact basilar arteries and compared that expression to that in cultured smooth muscle so that we can identify particular isoforms in which translocation, and hence activation, may be further examined.

The expression of PKC isoforms in basilar artery was found to differ from that of cultured cells. It is conceivable that this difference in expression is due to the phenotypic change smooth muscle cells undergo when cultured. When smooth muscle cells are cultured they rapidly change into a non-contractile state due to loss of myosin filaments (Ross, 1971 and 1993). Such changes underscore the shift in the expression levels of cellular proteins that takes place when smooth muscle cells are cultured. It is conceivable that the expression of many proteins may be equally altered in these processes, including those of particular PKC isoforms. Another factor which may contribute to the perceived differences in the expression of PKC isoforms between cultured cells and intact arteries may be due to cross-reactivity of the PKC antibodies used. It has been reported that the antibodies directed against the α and β isoforms of PKC are capable of cross reacting with one another. In consideration of this evidence, clear identification of either the α or

β isoforms cannot be made. Therefore, any expression change observed between these two enzymes is, at best, uncertain. Nonetheless, these studies indicate that there are particular isoforms of PKC which are expressed regardless of sample. These types are PKC-α, -ε, and -λ. From previous contractility studies utilizing specific inhibitors of PKC, two of these enzymes, PKCα and PKCε, appear to participate in vascular contractility stimulated by OxyHb and ET-1. Thus, to further explore the potential involvement of PKCα and PKCε in vascular contraction we have examined the translocation of these two isozymes to cellular membrane fractions following stimulation with OxyHb.

Exposure of cultured CVSMC to OxyHb for a period of time corresponding to maximum tonic contraction was found to yield a statistically significant increase in membrane bound PKCα. This evidence suggests that the α isoform of PKC is a likely candidate to be involved in prolonged vasoconstriction due to OxyHb and therefore is also likely to be involved in cerebral vasospasm. In addition, the translocation of PKCε in response to OxyHb was examined and was also found to be significantly higher in membrane fractions in comparison to untreated controls. This evidence suggests that PKCε may also be involved in the development of vasoconstriction induced by OxyHb. The results taken together are consistent with those obtained with the specific inhibitors of PKC, Ro32-0432 and Gö 6976. These observations confirm that the classical isoforms of PKC are likely to play critical roles in the development of vascular tone in response to OxyHb and that the novel, Ca^{2+} independent PKCε isoform, may also be involved, although it appears to have a less dominant role than that of α.

4.2 VASORELAXANT EFFECTS OF TAMOXIFEN

The present studies have demonstrated that tamoxifen (TAM), an antiestrogen compound widely used in the treatment of breast cancer, can reverse sustained contraction induced by OxyHb and ET-1. These results indicate that TAM may be of potential use in treating cerebral vasospasm following SAH. Consistent with this possibility, the IC_{50} values for TAM against OxyHb and ET-1 stimulated contraction, correlate with the therapeutic plasma concentration of TAM (0.5-1 μM) achieved in the treatment of breast cancer patients, indicating a clinically achievable relaxant effect for TAM (Bratherton *et al.*, 1984). In addition to the inhibition of contraction induced by OxyHb and ET-1, TAM also effectively attenuated sustained contraction induced by PMA, a direct activator of PKC. These observations suggest that the effects of tamoxifen may be mediated, at least in part, via inhibition of PKC. This suggestion is further supported by the observation that OxyHb and PMA-stimulated translocation of PKC activity from cytosol to plasma membrane fractions was abolished by TAM. TAM, a highly lipophilic molecule, has been shown to interact with the plasma membrane and decrease fluidity which may, in turn, inhibit the action of membrane enzymes such as PKC (Wiseman, 1994). Thus, it is conceivable that TAM interferes with PKC translocation, a suggestion for which there is some experimental evidence (Cheng *et al.*, 1998). It is also possible that TAM interferes with the hydophobic interactions between the regulatory domain of PKC and PS, a lipid co-factor necessary for activation (Walsh *et al.*, 1996)

Under this scheme the relaxant effect of TAM occurs independently of the estrogen receptor (Wickman and Vollrath, 2000). However, TAM binds to the estrogen

receptor in the nanomolar range, while the Ki values for TAM mediated inhibition of purified PKC were reported to be between 5-100 μM. Therefore the effects of estrogen receptor antagonism by tamoxifen cannot be excluded (Cheng et al., 1998). Nonetheless, estradiol, when applied to isolated samples, was devoid of any vasoaction while TAM retained its relaxant properties, suggesting that these two compounds, in the context of smooth muscle contraction, do not antagonize one another.

The observation that TAM was more effective against OxyHb than against PMA-mediated vasoconstriction suggests that, in addition to PKC inhibition, TAM may produce vasodilation through other mechanisms. This possibility is supported by the finding that TAM inhibited contraction mediated by depolarizing concentrations of potassium chloride, which generate contraction by promoting calcium influx through voltage dependent calcium channels (VDCC). Further evidence is provided by electrophysiological studies which have shown that TAM inhibits the voltage dependent calcium current in rat aortic smooth muscle (Song *et al.*, 1996). However, as previously mentioned, calcium channel antagonists are ineffective at reversing vasospasm, so that L-type channels are believed not to be involved in the development of this disorder (Tettenborn and Dycka, 1990). Therefore, the effectiveness of TAM in ameliorating vasospasm would not be ascribed to inhibition of L-type calcium channels. Yet, L-type channel antagonists are effective at preventing cerebral ischemia and infarction following vasospasm. Thus, TAM-induced inhibition of VOCC may prove to be effective at limiting calcium overload and preventing neuronal death in addition inducing vascular relaxation by other mechanisms. TAM has also been shown to have a number of other effects including antagonism of nonselective ion channels and calmodulin, and inhibition

of myosin light chain kinase (Mcgregor and Jordan, 1998 ; Lam, 1984 ; Friedman *et al.*, 1998), which may be the potential sites of action of this agent in smooth muscle cells. Therefore, it is likely that the effectiveness of TAM in attenuating OxyHb-mediated contraction arises from multiple effects of this agent.

The results of the present studies indicate that TAM in clinically effective concentrations is able to prevent the contractile action of OxyHb and suggest that this effect may be mediated, in part, by inhibition of PKC. These observations support the therapeutic potential of TAM in preventing or reversing vasospasm and could further the development of effective pharmacotherapy in this disorder.

4.3 VASORELAXANT EFFECTS OF THE AMINOGLYCOSIDE ANTIBIOTICS

The present studies demonstrate that gentamicin and the structurally related aminoglycosides are capable of attenuating ET-1 effects in cerebral arteries and CVSMC. This and previous studies (Nessim, 1997 ; Gergawy *et al.*, 1998) have shown that the aminoglycoside antibiotics concentration-dependently reverse sustained vasoconstriction due to OxyHb in isolated cerebrovascular preparations. The relaxant effect of these antibiotics against OxyHb (10 μM) and ET-1 (0.5 nM) was prolonged, suggesting that the aminoglycosides may be efficacious in reversing sustained vasospasm. In addition, gentamicin effectively attenuated PMA-induced sustained contraction in calcium free medium, suggesting that a potential mechanism of action of the aminoglycosides may be via inhibition of PKC activation. Further support is provided by the observations that the aminoglycosides directly inhibited the membrane association, and thus the activation, of PKC in CVSMC exposed to ET-1 and PMA. In addition, protein phosphorylation

mediated by phorbol esters in cultured epithelial cell has been shown to be prevented by the aminoglycosides (Hagiwara *et al.*, 1988). Collectively this evidence suggests that the aminoglycoside antibiotics inhibit the activation of PKC.

The mechanism by which aminoglycosides may mediate vascular relaxation is unclear but it is likely via interaction of these compounds with acidic, negatively charged phospholipids in the plasma membrane. The aminoglycosides are polybasic due to their protonated amino groups and anionic phospholipids of the plasma membrane are prime targets for a charge interaction with these compounds (Lullmann and Vollmer, 1982). Neomycin has been shown to bind selectively to phosphoinositides to interfere with the enzymatic hydrolysis of these lipids by PLC isoforms (Schibeci and Schacht, 1977). ET-1-mediated ET_A receptor activation initiates vascular constriction via Gq protein coupling to PLC_β. Therefore inhibition of PLC_β by the aminoglycosides is a conceivable mechanism of action of these agents. However, the activation of PLC_β and hydrolysis of PIP_2 is transient and following 5 min of stimulation the intracellular concentration of IP_3 is not different from that of control (Berridge, 1993). This observation suggests that the prolonged contractile response elicited by ET-1 is unlikely to be mediated by the activation of PLC_β alone. Thus aminoglycoside inhibition of PLC_β may not be the only mechanism of action whereby these compounds induce vascular relaxation. The binding of the aminoglycosides to inositol phospholipids may also inhibit the activation of PLD, an enzyme believed to be responsible for sustained vasoconstriction (Asano and Matsui, 1999). Inhibition of PLD would limit the prolonged production of DAG derived from PC breakdown. This process is believed to be mediated by the binding of the aminoglycosides to PIP_2, an important cofactor in PLD activation (Schmidt *et al.*, 1996).

It is conceivable that the aminoglycosides also bind PS, thus interfering with the activation of PKC. Phosphatidylserine is a necessary cofactor for PKC activation (Newton, 1997). Therefore aminoglycoside binding to this phospholipid may attenuate the ability of the enzyme to respond to activation by DAG and limit the phosphorylation of target proteins which are necessary to maintain smooth muscle contraction. Nevertheless, it is clear that the aminoglycoside antibiotics possess the ability to inhibit the activation of PKC, potentially through multiple mechanisms. However, additional studies are required to understand the cellular consequences of aminoglycoside interactions.

Although these studies provide strong evidence for PKC inhibition as the primary mechanism for the vasodilatory effects of the aminoglycosides the possibility of alternative modes of action cannot be discounted. Aminoglycoside antibiotics have been recognized to inhibit a variety of calcium channels, including voltage dependent L-type calcium channels (Langton *et al.*, 1996), and calcium release-activated channels known to be stimulated by capacitative calcium entry (Kim, 1999). In addition, the aminoglycosides have also been shown to attenuate sustained increases in intracellular calcium stimulated with hemoglobin (Gergawy *et al.*, 1998). ET-1 stimulates calcium influx mediated by a variety of voltage dependent and independent calcium channels in smooth muscle cells (Nakajima *et al.*, 1996 ; Iwamuro *et al.*, 1999). Therefore it is likely that the aminoglycosides relax vasoconstriction stimulated by ET-1 through multiple actions. Although L-type calcium channels do not appear to play a role in the pathogenesis of vasospasm, aminoglycoside-induced inhibition of calcium influx via other pathways may lead to an attenuation of cerebral artery constriction during

vasospasm. In addition, the ability of the aminoglycosides to inhibit calcium influx via voltage-operated calcium channels may have neuroprotective effect during cerebral vasospasm. As previously discussed, activation of L-type channels may be important in the development of delayed ischemic neurologic deficit resulting from vasospasm.

The present studies demonstrate that the aminoglycoside antibiotics are effective inhibitors of vasoconstriction and maintain vascular relaxation over a prolonged period of time. These compounds are efficacious when administered after the initiation of vasoconstriction, which fulfills a prerequisite for an anti-spastic agent, as treatment is almost always started after the onset of clinical spasm. Although the aminoglycosides may induce relaxation via multiple mechanisms these studies show that this effect is, at least in part, due to inhibition of PKC activation. It is now recognized that the pathogenesis of cerebral vasospasm is multifactorial. Thus an advantage may exist for the aminoglycoside and related compounds over specific antagonists or signal transduction inhibitors in the management of cerebral vasospasm. The implication of such observations is that, for successful management of vasospasm, agents may need to interfere with common biochemical pathways leading to pathological constriction. This observation suggests that the aminoglycosides may be the compounds to lead the search for pharmacological means of reversing the development of vasospasm and prevent the associated morbidity and mortality.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

The present studies support the hypothesis that PKC plays a key role in cerebrovascular contraction induced by OxyHb and ET-1, the primary spasmogens

implicated in the pathogenesis of cerebral vasospasm. To our knowledge these studies are the first to demonstrate that activation of PKC, in particular PKCα and PKCε isoforms, is a critical component of OxyHb and ET-1-mediated sustained cerebrovascular vasoconstriction characteristic of vasospasm. These observations implicate the activity of PKC in the pathological processes leading to sustained vasospasm following SAH. In addition, these studies show that the aminoglycoside antibiotics and TAM are effective inhibitors of vascular constriction induced by both OxyHb and ET-1. The actions of TAM and the aminoglycosides appear to be mediated, at least in part, through the inhibition of PKC. The relaxant effects of these compounds may be of potential benefit in the management of cerebral vasospasm.

Although PKC has been shown in this study to play a key role in OxyHb and ET-1-induced cerebrovascular constriction, the signaling events initiated by these compounds are far from clear. Investigation of the cellular signaling mediated by these spasmogenic factors in cerebrovascular smooth muscle remains an open field of investigation. More extensive studies focusing on molecular targets of PKC isoforms would provide new information about the mechanism of smooth muscle contraction. Better understanding of these signaling processes may also be achieved by examining the role of MAPK in contraction initiated by OxyHb and ET-1. This goal could be accomplished by examining: the time course of MAPK activation, the proteins which become phosphorylated, and the functional consequences of its activation. As suggested earlier, studies using animal models of cerebral vasospasm could provide some additional answers to the pertinent questions that arise from this research. Animal experiments would permit the analysis of signaling at various stages of disease progression and in this

way, may provide some clues as to the nature of the compounds involved in persistent vasoconstriction. These experiments may further aid the search for a pharmacological means of reversing this disorder.

While these studies show that TAM and the aminoglycosides reverse OxyHb and ET-1 induced vasoconstriction, the efficacy of these compounds against vasospasm has yet to be tested. Thus, it would be desirable to test these compounds in an animal model of vasospasm so that we can get a clear picture of their therapeutic potential.

CHAPTER 5:

5.0 REFERENCES

Adam LP., Gapinski C., and Hathaway D. Phosphorylation sequences in h-caldesmon from phorbol ester-stimulated canine aortas. FEBS Lett. 303: 223-226. 1995.

Adam LP. and Hathaway D. Identification of mitogen-activated protein kinase phosphorylation sequences in mammalian h-caldesmon. FEBS Lett. 322: 56-60. 1993.

Adams HP., Goodman FR., and Weiss G. Alteration of contractile function and calcium movements in vascular smooth muscles by gentamicin and other aminoglycoside antibiotics. Antimicrob. Agents Chemother. 5: 640-646. 1974.

Adams HP. Prevention of brain ischemia after aneurysmal subarachniod hemorrhage. Neurol. Clin. N. Am. 10: 251-268.1992.

Aitken A., Lippman M., Kasid A., and Schoenberg D. Relationship between the expression of estrogen-regulated genes and estrogen-stimulated proliferation of MCF-7 mammary tumor cells. Cancer Res. 45: 2608-2615. 1985.

Allan G. and Bahr A. Cerebral arterial spasm: part 10; Reversal of acute and chronic spasm in dogs with orally administered nifedipine. Neurosurgery 4: 43-47. 1979.

Allan G., Ahn H., Preziosi T., Battye R., Boone S., Chou S., Kelly D., Weir BKA., Crabbe RA., Lavik P., Rocnbloom SB., Dorey F., Ingram C., Mellits DF., Bertsch L., Boisvert D., Hundley M., Johnson RK., Strom J., and Transou C. Cerebral arterial spasm, a controlled trial of nimodipine in patients with subarachnoid hemorrhage. N. Engl. J. Med. 308: 619-624. 1983.

Arai H., Hori S., Aramori I., Ohkubo H., and Nakanishi. Cloning and expression of cDNA encoding an endothelin receptor. Nature 348: 730-732. 1990.

Aramori I., and Nakanishi S. Coupling of two endothelin receptor subtypes to differing signal transduction in transfected Chinese hamster ovary cells. J. Biol. Chem. 267: 12468-12474. 1992.

Asano T., Ikegaki I., Suzuki Y., Satoh S., and Shibuya M. Endothelin and the production of cerebral vasospasm in dogs. Biochem. Biophys. Res. Commun. 159: 1345-1351. 1989.

Asano T., and Matsui T. Antioxidant therapy against cerebral vasospasm following aneurysmal subarachnoid hemorrhage. Cell. Molec. Neurobiol. 19: 31-44. 1999.

Berridge MJ. Inositol trisphosphate and calcium signaling. Nature 361: 315-325. 1993

Berridge MJ., and Irvine RF. Inositol phosphates and cell signaling. Nature 341: 197-205. 1989.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72: 248-254.1976.

Braughler J., Pregenzer J., Chase R., Duncan L., Jacobsen E., and McCall J. Novel 21-aminosteroids as potent inhibitors of iron-dependent lipid peroxidation. J. Biol. Chem. 262: 10438-10440. 1987.

Braughler J., Burton P., Chase R., Pregenzer J., Jacobsen E., VanDoornik F., and Tustin J. Novel membrane localized iron chelators as inhibitors of iron dependent lipid peroxidation. Biochem. Pharmacol. 37: 3863-3860. 1988.

Braughler J., and Pregenzer J. The 21-aminosteroid inhibitors of lipid peroxidation: reactions with lipid peroxyl and phenoxyl radicals. Free Radicals Biol. Med. 7: 125-130. 1989.

Bratherton D., Brown C., Buchanan R., Hall, V., Pillers E., Wheeler T., and Wiliams C. A Comparison of two doses of tamoxifen (Nolvadex) in postmenopausal women with advanced breast cancer: 10mg versus 20mg. Br. J. Cancer. 50: 199-205. 1984.

Butler A., Flintey F., and Williams D. NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron nitrosyls in biology: a chemist perspective. Trends in Pharmacol. Sci. 16: 18-23. 1995.

Carney D., Scott D., Gordon E., and LaBell E. Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. Cell 42: 479-488. 1985.

Cazaubon S., Bornancin F., and Parker P. Threonine-497 is a critical site for permissive activation of protein kinase C α. Biochem. J. 301: 443-448. 1994.

Chapman G., and Wallace H. Spermine prevents lipid peroxidation by essential fatty acids in human breast cancer cells. Biochem. Soc. Trans. 22: 401s. 1994.

Cheng A., Chuang S., Fine R., Yeh K., Liao C., Lay J., and Chen D. Inhibition of the membrane translocation and activation of protein kinase C, and potentiation of doxorubicin-induced apoptosis of hepatocellular carcinoma cells by tamoxifen. Biochem. Pharmacol. 55: 523-531. 1998.

Clarke R., van den Berg H., and Murphy R. Reduction of the membrane fluidity of human breast cancer cells by tamoxifen and 17β-estradiol. J. Natl. Cancer Inst. 82: 1702-1705. 1990.

Collins E., Walsh M., and Morgan K. Contraction of single vascular smooth muscle cells by phenylephrine at constant $[Ca^{2+}]_i$. Am. J. Physiol. 262: H754-H762. 1992.

Cook D., Weir B., Okwasaba F., and Kreuger C. Effects of hemoglobin on smooth muscle. Proc. Western Pharmacol. Soc. 22: 429-434. 1979.

Cook D. and Vollrath B. Free radicals and intracellular events associated with cerebrovascular spasm. Cardiovascular Res. 30: 493-500. 1995.

Cosentino F. and Katusic Z., Does endothelin-1 play a role in the pathogenesis of cerebral vasospasm? Stroke 25: 904-908.1994.

Doi M., Allen B., Walsh M., Kauya H., Weir B., and Cook D. Involvement of calcium binding protein in the development of arterial spasm. In: Finlay J.M., ed. Cerebral Vasospasm: Proceedings of the V th international conference on cerebral vasospasm. Amsterdam: Elsevier Science Publishers 109-112. 1993.

Ecker A., Riemnshneider P., Arteriographic demonstration of spasm of the intracranial arteries with special reference to saccular arterial aneurysms. J. Neurosurg. 8: 660-667. 1951.

Edward K., Laughton C., and Neidle S. A molecular modeling study of the interactions between the antiestrogen drug tamoxifen and several derivatives, and the calcium-binding protein clamodulin. J. Med. Chem. 35: 2753-2761. 1992.

Espinosa F., Weir B., Overton T., Castor W., Grace M., and Boisvert D. A randomized placebo-controlled double-blind trial of nimodipine after SAH in monkeys. Part 1: Clinical and radiological findings. J. Neurosurg. 60: 1167-1175. 1984.

Fasolato C., Innocenti B., and Pozzan T. Receptor-operated Ca^{2+} influx: How many mechanisms for how many channels? TiPS. 15: 77-83. 1994.

Fein F., Flor W., and Cohan S. Sequential changes of vascular ultrastructure in experimental cerebral vasospasm. J. Neurosurg. 41: 49-58. 1974.

Findlay JM., Weir BKA., Kanamaru K., Espinosa F. Arterial wall changes in cerebral vasospasm. Neurosurgery. 25: 736-746. 1989.

Findlay JM., Macdonald R., and Weir BKA. Current concepts of pathophysiology and management of cerebral vasospasm following aneurysmal subarachnoid hemorrhage. Cerebrovasc. Brain Metab. Rev. 3: 336-360. 1991.

Friedman Z. Recent advances in understanding the molecular mechanisms of tamoxifen action. Cancer Invest. 16: 391-396. 1998.

Furchgott R., Zawadzki J. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373-376. 1980.

Gergawy M., Vollrath B., and Cook D. The mechanism by which aminoglycoside antibiotics cause vasodilation of canine cerebral arteries. Br. J. Pharmacol. 125: 1150-1157. 1998.

Gibson D., Gottardis M., and Jordan V. Sensitivity and insensitivity of breast cancer to tamoxifen. J. Steroid Biochem. Mol. Biol. 37: 765-779. 1990.

Gschwendt M., Kittstein W., and Marks F. Protein kinase C activation by phorbol esters : do cysteine-rich regions and pseudosubstrate motifs play a role? TIBS. 16: 167-169. 1991.

Gutteridge JMC., Halliwell B. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem. Sci. 15:129-135. 1990.

Hagiwara M., Inagaki M., Kanamura K., Ohta H., and Hidaka H. Inhibitory effects of aminoglycosides on renal protein phosphorylation by protein kinase C. J. Pharmacol. Exp. Ther. 244: 355-360. 1988.

Hahn A,. Resink T., Scott-Burden T., Powell J., Dohi, Y., and Buhler F. Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function. Cell Regulation 1: 649-659. 1990.

Hall E., McCall J. Antioxidant action of lazaroids. In: Packer L., ed. Methods in Enzymology: Oxygen Radicals in Biological Systems, Part C. San Diego: Academic Press, 560-567. 1994.

Halliwell B., and Gutteridge J. Oxygen free radicals and iron in relation to biology and medicine: new problems and concepts. Arch. Biochem. Biophys. 246: 501-514. 1986.

Hamann G., Isenberg E., Strittmatter M., and Schimrigk K. Absence of elevation of big endothelin in subarachnoid hemorrhage. Stroke 24: 383-386. 1993.

Hemric ME., and Chalovich J. Effect of caldesmon on the ATPase activity and the binding of smooth and skeletal myosin subfragments to actin. J. Biol. Chem. 263: 1878-1885. 1988.

Himpens B., Kitazawa T., and Somlyo A.P. Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. Pflugers Archiv. Eur. J. Physiol. 417: 21-28.1990.

Hongo K., Kassel N., Nakagomi M., Sasaki T., Tsukahara T., Ogawa H., Vollmer D., and Lehman R. Subarachnoid hemorrhage inhibition of endothelium-derived relaxing factor in rabbit basilar artery. J. Neurosurg. 69: 247-253. 1988.

Horgan K., Cooke E., Maurice B., Hallett M., and Mansel R. Inhibition of protein kinase C mediated signal transduction by tamoxifen. Biochem. Pharmacol. 35: 4463-4465. 1986.

Horowitz A., Clement-Chomienne O., Walsh M., Tao T., Katsuyama and Morgan K. Effects of calponin on force generation by single smooth muscle cells. Am. J. Physiol. 270: H1858-H1863. 1996.

Horowitz A., Menice CB., Laport R., and Morgan K. Mechanisms of Smooth Muscle Contraction. Physiological Reviews. 76: 967-1003. 1996.

Hostetler K. and Hall L. Inhibition of kidney lysosomal phospholipase A and C by aminoglycoside antibiotics: possible mechanism of aminoglycoside toxicity. Proc. Natl. Acad. Sci. USA. 79: 1663-1667. 1982.

Hughes J. Schianchi P. Cerebral artery spasm: a histological study at necropsy of the blood vessels in cases of subarachnoid hemorrhage. J. Neurosurg. 48: 515-525. 1978.

Hunley T., Iwasaki S., Homma T., and Kon V. Nitric oxide and endothelin in pathophysiological settings. Pediatr. Nephrol. 9: 235-244. 1995.

Ide K., Yamakawa K., Nakagomi T., Sasaki T., Saito I., Kutihara H., Yosizumi T., Yazaki Y., and Takakura K. The role of endothelin in the pathogenesis of vasospasm following subarachnoid hemorrhage. Neurol. Res. 11: 101-104. 1989.

Ignarro LJ., Buga GM., Wood K., Byrns R., Chaudhri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA 1987. 54: 9625. 1987.

Inoue A., Yanagisawa M., Kimura S., Kasuya Y., Miyauchi T., and Goto K. The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. USA. 86: 2863-2867. 1990.

Itoh S., Sasaki T., Ide K., Ishikawa K., Nishikibe M., and Yano M. A novel endothelin ET_A receptor antagonist BQ-485 and its preventative effect on experimental vasospasm in dogs. Biochem. Biophys. Res. Commun. 195: 969-975. 1993.

Itoh S., Sasaki T., Asai A., and Kuchino Y. Prevention of delayed vasopasm by an endothelin ET_A receptor antagonist BQ-123: Change of ET_A receptor expression in a canine subarachnoid hemorrhage model. J. Neurosurg. 81: 759-764. 1994.

Iwamuro Y., Miwa S., Zhang X-F., Minowa T., Enoki T., Okamoto Y., Hasegawa H., Furutani H., Okazawa M., Ishikawa M., Hashimoto N., and Masaki T. Activation of three types of voltage independent Ca^{2+} channel in A7r5 cells by endothelin-1 as revealed by a novel Ca^{2+} channel blocker LOE 908. Br. J. Pharmacol. 126: 1107-1114. 1999.

Kamii H., Kato I., Kinouchi H., Chan P., Epstein C., Akabane A., Okamoto H., and Yoshimoto T. Amelioration of vasospasm after subarachnoid hemorrhage in transgenic mice overexpressing CuZn-Superoxide dismutase. Stroke 30: 867-872. 1999.

Kamiyama K., Okada H., and Suzuki J. The relation between cerebral vasospasms and superoxide. Application of an experimental model of vasospasm induced by oxyhemoglobin in cats. Neurol. Med. Chir. (Tokyo). 21: 201-209. 1981.

Kassell NF., Drake CG. Timing of aneurysmal surgery. Neurosurgery. 10: 514-519. 1982.

Kassell NF., Sasaki T., Colohan AR., Nazar G. Cerebral vasospasm following aneurysmal subarachnoid hemorrhage. Stroke 16: 562-572. 1985.

Kassell NF., Torner J., and Jane J. The international cooperative study on the timing of aneurysm surgery. In Auer L., and Kassel N (eds). Timing of aneurysmal surgery, Hawthorne N., and Degruyter. 227-278. 1985.

Kassell NF. The role of vasospasm in overall outcome from aneurysmal subarachnoid hemorrhage. in Findlay J (ed). Cerebral vasospasm. pp27. 1993.

Kassell NF., Torner JC., Haley EC.; Jane JA., Adams HP., and Kongable G.L. The international cooperative study on the timing of aneurysm surgery. I.Overall management results. J. Neurosurg. 73: 18-36. 1990.

Kassell NF., Haley E., Apperson-Hansen C., Alves W., and the participants. Randomized, double blind, vehicle controlled trial of trilizad mesylate in patients with aneurysmal subarachnoid hemorrhage: cooperative study in Europe, Australia, and New Zealand. J. Neurosurg. 84: 221-228. 1996.

Kasuya H., Weir B., White D., and Stefansson K. Mechanism of oxyhemoglobin-induced release of endothelin1- from cultured vascular endothelial cells and smooth-muscle cells. J. Neurosurg. 79: 892-898. 1993.

Kemp B., Pearson R., Guerriero V., Bagchi I., and Means A. The calmodulin binding domain of chicken smooth muscle myosin light chain kinase contains a pseudosubstrate sequence. J. Biol. Chem. 264: 15843-15849. 1987.

Khalil R., and Morgan K. PKC mediated redistribution of mitogen activated protein kinase during smooth muscle activation. Am. J. Physiol. 265: C406-C411. 1993.

Kim P., Schini V., Sundt T., and Vanhoutte P. Reduced production of cGMP underlies the loss of endothelin-dependent relaxations in the canine basilar artery after subarachnoid hemorrhage. Circ. Res. 70: 248-256. 1992.

Klee, C. Calmodulin: structure-function relationships. Calcium and Cell Function. Vol.1. Academic press, New York. Pp.59-78. 1980.

Kobayashi H., Hayashi M., Kobayashi S., Kabuto M., Handa Y., and Kawano H. Effect of endothelin on the canine basilar artery. Neurosurg. 27: 357-361. 1990.

Koide T., Neichi T., Takato M., Matsushita H., Sugioka K., Nakano M., Hata S. Possible mechanisms of 15-hydroperoxyarachidonic acid-induced contraction of the canine basilar artery *in vitro*. J. Pharm. Exp. Ther. 221: 481-488. 1982.

Lam H. Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem. Biophys. Res. Commun. 118: 27-32. 1984.

Langton P., Farley R., and Everitt D. Neomycin inhibits K^+-induced force and Ca^{2+} channel current in rat arterial smooth muscle. Pflugers Arch. Eur. J. Physiol. 433: 188-193. 1996.

Lassegue B., Alexander R., Clark M., Akers M., and Griendling K. Phosphatidycholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth muscle cells. Biochem. J. 292: 509-517. 1993.

Lopes M., Vale M., Carvalho A. Ca^{2+}-dependent binding of tamoxifen to calmodulin isolated from bovine brain. Cancer Res. 50: 2753-2758. 1990.

Lullmann H., and Vollmer B. An interaction of aminoglycoside antibiotics with Ca^{2+} binding to lipid monolayers and to biomembranes. Biochem. Pharmacol. 31:3769-3773. 1982.

Lutrell L., Daaka Y., and Lefkowitz R. Regulation of tyrosine kinase cascades by G-protein coupled receptors. Curr. Opin. in Cell Biol. 11: 177-183. 1999.

Macdonald R.L., and Weir BKA. A review of hemoglobin and the pathogenesis of cerebral vasospasm. Stroke 22: 971-982. 1991.

Macdonald R.L., Weir BKA., Runzer T., Grace M., and Poznansky M. Effect of intrathecal superoxide dismutase and catalase in oxyhemoglobin-induced vasospasm in monkeys. Neurosurgery 30: 529-539. 1992.

Macdonald RL. Cerebral vasospasm. Neurosurgery 5: 73-97. 1995.

Macgregor J. and Jordan V. Basic guide to the mechanisms of antiestrogen action. Pharmacol. Rev. 50: 151-196. 1998.

Marche P., Koutouzov S., and Girard A. Impairment of membrane phosphoinositide metabolism by aminoglycoside antibiotics: streptomycin, amikacin, kanamycin, dibekacin, gentamicin, and neomycin. J. Pharmacol. Exp. Ther. 227:415-420. 1983.

Marquardt B., Firth D., and Stabel S. Signaling from TPA to MAP kinase requires protien kinase C, Raf, and MEK: reconstitution of the signaling pathway in vitro. Oncogene 9: 3213-3218. 1994.

Martin W., Villani G., Jothianandan D., and Furchgott R. Selective blockade of endothelium-dependent and glyceryl trinitrate induced relaxation by hemoglobin and methylene blue. J. Pharm. Exp. Ther. 232: 708-716. 1985.

Martiny-Baron G., Kazanietz M., Mischak H., Blumberg P., Kochs G., Hug H., Marme D., and Schachtele C. Selective inhibition of protein kinase C isozymes by the Indolocarbazole Gö 6976. J. Biol. Chem. 268: 9194-9197. 1993.

Matsui T., Sugawa M., Takuwa Y., and Asano T. Activation of protein kinase C mediated contractile system in the canine basilar artery undergoing chronic vasospasm. Stroke 22: 1183-1187. 1991.

Matsui T., Takuwa Y., Nagafuji K., Kaizu H., and Asano T. Possible role of protein kinase C-dependent smooth muscle contraction in the pathogenesis of chronic cerebral vasospasm. J. Cereb. Blood Flow Metab. 11: 143-149. 1990.

Matsumam Y., Ikegawa R., Tsukahara Y., Takaoka M., and Morimoto S. Conversion of big endothelin-1 to endothelin-1 by two types of metalloproteinases derived from porcine aortic endothelial cells. FEBS lett. 272: 166-170. 1990.

Matsumara Y., Ikegawa R., Suzuki Y., Takaoka M., Uchida T., Kido H., Shinyama H., Hayashi K., Watanabe M., and Moromoto S. Phosphoramidone prevents cerebral vasospasm following subarachnoid hemorrhage in dogs: The relationship to endothelin-1 levels in cerebrospinal fluid. Life Sci. 49: 481-484.1991.

Mayberg M., Okada T., and Bark D. The significance of morphological changes in cerebral arteries after subarachnoid hemorrhage. J. Neurosurg. 72: 626-633. 1990.

Megyesi JF., Vollrath B., Cook D., and Findlay JM. In vivo animal models of cerebral vasospasm: a review. Neurosurgery 46: 448-460. 2000.

Miki M., Walsh M., and Hartshorne D. The mechanism of inhibition of the actin-activated myosin MgATPase by calponin. Biochem. Biophys. Res. Commun. 187: 867-871. 1992.

Minami N., Tami E., Maeda Y., Yamaura I., and Fukami M. Effects of inhibitors of protein kinase C and calpain in experimental delayed cerebral vasospasm. J. Neurosurg. 76: 111-118. 1992.

Nakadate T., Jeng, A., and Blumberg, PM. Comparison of protein kinase C functional assay to clarify mechanisms of inhibitor action. Biochem. Phamacol. 37: 1541-1545. 1988.

Nakajima T., Hazama H., Hamada E., Wu S-N., Igarashi K., Yamahita T., Seyama Y., Omata M., and Kurachi Y. Endothelin-1 and vasopressin activate Ca^{2+}-permeable non-selective cation channels in aortic smooth muscle cells: Mechanism of receptor-mediated Ca^{2+} influx. J. Mol. Cell. Cardiol. 28:707-722. 1996.

Nessim M. Effects of aminoglycoside antibiotics on intracellular processes in cerebral vasospasm. U of A. Dept Pharmacol. M.Sc. Thesis. 1997.

Newton A. Protein kinase C: Structure, function, and regulation. J. Biol. Chem. 270: 28495-28498. 1995.

Newton A. Regulation of protein kinase C. Curr. Opin. in Cell Biol. 9: 161-167. 1997

Nieri H., Hamada K., Shoubo M., Sogabe K., Notsu Y. and Ono T. An endothelin ET_A receptor antagonist Fr 139317 ameliorates vasospasm in dogs. Life Sci. 52:1869-1674. 1993.

Nishizawa S., Nezu N., and Uemura K. Direct evidence of a key role of protein kinase C in the development of vasospasm after subarachnoid hemorrhage. Stroke 18: 482-489. 1992.

Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607-614. 1992.

Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9: 484-496. 1995.

Noseworthy T., Weir BKA., Boisvert D., Espinosa F., Overton T., and Marshal M. Effect of reserpine-kanamycin treatment on chronic vasospasm after platelet-enriched subarachnoid hemorrhage in primates. Neurosurgery 14: 193-197. 1984.

Nosko M., Weir BKA., Krueger C., Cook D., Norris S., Overton T., and Boisvert D. Nimdipine and chronic vasospasm in monkeys: Part1: Clinical and radiological findings. Neurosurgery 16: 129-136. 1985.

O'Brian C., Liskamp R., Soloman D., and Weinstein B. Inhibition of protein kinase C by tamoxifen. Cancer Res. 45: 2462-2465. 1985.

O'Brian C., Ward N., Anerson B: Role of specific interactions between protein kinase C and triphenylethylenes in inhibition of the enzyme. J. Natl. Cancer. Inst. 80: 1628-1633, 1988.

Ohlstein E. and Storer B. Oxyhemoglobin stimulation of endothelin production in cultured endothelial cells. J. Neurosurg. 77: 274-278.1992.

Onoda K., Shigeki O., Ogihara K., Shiota T., Asari S., Ohmoto T., and Nimomiya Y. Inhibition of vascular contraction by intracisternal administration of preproendothelin-1 mRNA antisence oligoDNA in a rat experimental vasospasm model. J. Neurosurg. 85: 846-852. 1996.

Onoue H., Kaito N., Akiyama M., Tomii M., Tokudome S., and Abe T. Altered reactivity of human cerebral arteries after subarachnoid hemorrhage. J. Neurosurg. 83: 510-515. 1995.

Orr J. and Newton A. Requirement for negative charge on 'activation loop' of protein kinase C. J. Biol. Chem. 269: 27715-27718. 1994.

Palmer RM., Ashton D., and Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333: 664 . 1988.

Pearson R., Wettenhall A., Means A., Hartshorne D., and Kemp B. Autoregulation of enzymes by pseudosubstrate prototypes: myosin light chain kinase. Science 241: 970-973. 1988.

Peterson J., Canida G., Spanos A., and Zervas N. The calmodulin antagonist trifluoperazine provides mild prophylactic protection against cerebral vasospasm after subarachnoid hemorrhage, but no therapeutic value. Neurosurgery 72:767-774. 1989.

Pickard JD., Murray G., Illingworth R., Shaw M., Teasdale G., Foy P., Humphery P., Land D., Nelson R., and Richard P. The effect of nimodipine on cerebral infarction and outcome after subarachnoid hemorrhage: British aneurysm nimodipine trial. B.M.J. 298: 636-642. 1989.

Pickard JD., Nelson R., Martin JL.,Cerebral Vasospasm, in Crockard A., Hayward R., and Hoff JT. (eds): Neurosurgery. The scientific basis of clinical practice. Blackwell, Cambridge 1-38. 1992.

Pluta RM., Afshar J., Boock R., and Oldfield E. Temporal changes in perivascular concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin after subarachnoid hemorrhage. J. Neurosurg. 88: 557-561. 1988.

Randall M., Douglas S., and Hiley C. Vascular activities of ET-1 and some alanyl substituted analogues in resistance beds of the rat. Br. J. Pharmacol. 98: 685-699. 1989.

Rasmussen H., Takuwa Y., and Park S. Protein kinase C in the regulation of smooth muscle contraction. FASEB J. 1: 177-185. 1987.

Robinson M., and Teasdale G. Calcium antagonists in the management of subarachnoid hemorrhage. Cerebrovasc. Brain Metab. Rev. 2: 205-226. 1990.

Rohn T., Hinds T., and Vincenzi F. Inhibition of Ca^{2+}-pump ATPase and Na^{+}/K^{+}-pump ATPase by iron-generated free radicals. Biochem. Pharmacol. 51: 471-476. 1996.

Ross R. The smooth muscle cells. II. Growth of smooth muscle in culture and formation of elastic fibers. J. Cell. Biol. 50: 172-186. 1971.

Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801-807. 1993.

Rousseaux P., Sherpereel B., Bernard M., Graftieaux J., Guyot J. Fever and cerebral vasospasm in ruptured intracranial aneurysms. Surg. Neurol. 14: 459-465. 1980.

Roux S., Lofflers B., Gray G., Sprecher U., Clozel M. and Clozel J. The role of endothelin in experimental cerebral vasospasm. Neurosurgery 37: 78-86. 1995.

Rumenapp U., Schmidt M., Wahn F., Tapp E., Grannass A., and Jakobs K. Characteristics of protein kinase C and ADP-ribosylation factor-stimulated phospholipase D activities in human embryonic kidney cells. Eur. J. Biochem. 248: 407-414. 1997.

Saito A., Shiba R., Kimura S., Yanagisawa M., Goto K., and Masaki T. Vasoconstrictor response of large cerebral arteries of cats to endothelin, and endothelium-derived vasoactive peptide. Eur. J. Pharmacol. 162: 353-358. 1989.

Sako M., Nishihara J., Ohta S., Wang J., and Sakaki S., Role of protein kinase C in the pathogenesis of vasospasm following subarachnoid hemorrhage. J. Cereb. Blood Flow Metab. 13: 247-254. 1993.

Sakurai T., Yanagisawa M., and Masaki T. Molecular characterization of endothelin receptors. Trends in Pharmacol. Sci. 13: 103-107. 1992.

Sasaki T., Tanishima T., Asano T., Mayanagi Y., and Sano K. Significance of lipid peroxidation in the genesis of chronic vasospasm following rupture of an intracranial aneurysm. Acta Neurochir. Suppl. (Wein) 28: 536-540. 1979.

Sasaki T., Asano T., and Sano K. Cerebral vasospasm and free radical reactions. Neurol. Med. Chir. (Tokyo) 20: 145-153. 1980.

Sasaki T., Wakai S., Asano T., Watanabe T., Kirino T., Sano K. The effect of a lipid hydroperoxide of arachidonic acid on the canine basilar artery . An experimental study on cerebral vasospasm. J. Neurosurg. 54:357-365. 1981.

Sastrasinh M., Knauss T., Weinberg J., and Humes D. Identification of the aminoglycoside binding site in rat renal brush border membranes. J. Pharmacol. Exp. Ther. 222: 350-358. 1982.

Schibeci A. and Schacht J. Action of neomycin on the metabolism of phosphoinositides in the guinea pig kidney. Biochem. Pharmacol. 26: 1769-1774. 1977.

Schiffrin E. and Touyz R. Vascular biology of endothelin. J. Cardiovasc. Pharamacol. 32(suppl. 3): S2-S13. 1998.

Schlessinger J., and Ullrich A. Growth factor signaling by receptor tyrosine kinases. Neuron 9: 383-391. 1992.

Schmidt M., Rumenapp U., Nehls C., Ott S., Keller J., Eichel-Streiber C., and Jokobs K. Restoration of clostridium difficile toxin-B inhibited phoshpolipase D by phosphatidylinositol 4,5-bisphosphate. Eur. J. Biochem. 240:707-712. 1996.

Schmidt H., Lohmann S., and Walter U. The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. Biochim. Biophys. Acta. 1178: 153-175. 1993.

Seifert V., Lofflers B., Zimmermann M., Roux S., and Stolke D. Endothelin concentrations in patients with aneurysmal subarachnoid hemorrhage. J. Neurosurg. 82: 55-62. 1995.

Sekhar L., Heros R. Origin, growth and rupture of saccular aneurysms. Neurosurgery 8: 248-260. 1981.

Shibuya M., Suzuki Y., Sugita K., Saito S., Sasaki T., Takakura K., Nagata I., Kikuchi H., Takemae T., Hidaka H., and Nakashima. Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. J. Neurosurg. 76: 571-577. 1992.

Shinyama H., Uchida T., Kido H., Hayashi K., Watanabe M., Matsumura Y., Ikegawa R., Takaoka M., and Morimoto S. Phosphoramidon inhibits the conversion if intracisternally administered big endothelin-1 to endothelin-1. Biochem. Biophys. Res. Commun. 178: 24-30. 1991.

Shishido T., Suzuki Y., Qian L., Masaoka H., Niimi Y., and Hirakawa K. Effect of superoxide dismutase on cerebral vasospasm in a rabbit subarachnoid hemorrhage model. Findlay J., ed. Cerebral vasospasm : Proceedings of the V th international conference on cerebral vasospasm. Amsterdam: Elsevier Science Publishers 183-186. 1993.

Shirazi A., Iizuka K., Fadden P., Mosse C., Somlyo A.P., Somlyo A.V., and Haystead T. Purification and characterization of the mammalian myosin light chain phosphatase holoenzyme. J. Biol. Chem. 269: 31598-31606. 1994.

Simonson M., Wang Y., Johnes J., and Dunn M. Protein kinase C regulates activation of mitogen activated protein kinase and induction of protooncogene c-fos by endothelin-1. J. Cardiovasc. Pharmacol. 20: S29-S32. 1992.

Simpson A. and Ashley C. Endothelin evoked Ca^{2+} transients and oscillations in A-10 vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 163: 1223-1229. 1989.

Sobey C., Heistad D., and Faraci F. Effect of subarachnoid hemorrhage on dilation of basilar artery in vivo. Am. J. Physiol. 271: H126-H132. 1996.

Song J., Standley P., Zhang F., Joshi D., Gappy S., Sowers J., and Ram J. Tamoxifen (estrogen antagonist) inhibits voltage-gated calcium current and contractility in vascular smooth muscle from rats. J. Pharmacol. Exper. Ther. 277:1444-1453. 1996.

Steele J., Stockbridge N., Malijovic G., and Weir B. Free radicals mediate actions of oxyhemoglobin on cerebrovascular smooth muscle cells. Circ. Res. 68: 416-423. 1990.

Steinke D., Weir B., Findlay J., Tanabe T., Grace M., and Krushelnycky B. A trial of the 21-aminosteriod U74006F in a primate model of chronic cerebral vasospasm. Neurosurgery 24: 179-186. 1989.

Sun H., Kanawaru K., Masaaki I., Suzuki H., Kojima T., Waga S., Kureishi Y., and Nakano T. Myosin light chain phosphorylation and contractile proteins in canine two-hemorrhage model of subarachnoid hemorrhage. Stroke 29: 2149-2154. 1998.

Suzuki Y., Osuka K., and Noda A. Nitric oxide metabolites in the cisternal cerebral spinal fluid of patients with subarachnoid hemorrhage. J. Neurosurg. 70: 108-114. 1989.

Takahashi E. and Berk BC. MAP kinases and vascular smooth muscle function. Acta. Physiol. Scand. 164: 611-621. 1998.

Takanashi Y., Weir B., Vollrath B., Kasuya H., Macdonald R. L., and Cook D. Time course of changes in concentration of intracellular free calcium in cultured cerebrovascular smooth muscle cells exposed to oxyhemoglobin. Neurosurgery 30: 346-350. 1992.

Takayasu M., Suzuki Y., Shibuyta M., Asano T., Kanamori M., Okada T., Kageyama N., and Hidaka H. The effects of HA compound calcium antagonist on delayed cerebral vasospasm in dogs. J. Neurosurg. 65: 80-85. 1986.

Takuwa Y., Matsui T., Abe Y., Nagafuji T., Yamashita K., and Asano T. Alterations in protein kinase C activity and membrane lipid metabolism in cerebral vasospasm after subarachnoid hemorrhage. J. Cereb. Blood Flow Metab. 16: 409-415. 1993.

Tanabe Y., Sakata K., and Yamada H. Cerebral Vasospasm and ultrastructural changes in the cerebral arterial wall.: An experimental study. J. Neurosurg. 449: 229-238. 1978.

Tanishima T. Cerebral vasopasm: contractile activity of hemoglobin in isolated canine basilar arteries. J. Neurosurg. 53: 787-793. 1990.

Tettenborn D., Dycka J. Prevention and treatment of delayed ischemic dysfunction in patients with aneurysmal subarachnoid hemorrhage. Stroke 21(Suppl 4): 85-89. 1990.

Torner JC., Kassel N., and Haley E. The timing of surgery and vasospasm. Neurosurgery Clinics of North America 1: 355-347. 1990.

Touyz R., Tolloczko B., and Shiffrin E. Mesenteric vascular smooth muscle cells from SHR display increased Ca^{2+} responses to angiotensin II but not to endothelin-1. J. Hypertension 11: 663-637. 1994.

Tsuda T., Kawahara Y., Shii K., Koide M., Ishida Y., and Yokoyama M. Vasoconstrictor induced protein tyrosine phosphorylation in cultured vascular smooth muscle cells. FEBS Lett. 285: 44-48. 1991.

Turner AJ., and Murphy L. Molecular pharmacology of endothelin converting enzymes. Biochem. Pharmacol. 51: 91-102. 1996.

Varsos V., Liszczak T., Han D., Kistler J., Vielma J., Black P., Heros R., and Zervas N. Delayed cerebral vasospasm is not reversible by aminophylline, nifedipine, or papaverine in a "two hemorrhage" canine model. J. Neurosurg. 58:11-17. 1983.

Vollmer D., Kassel N., Hongo K., Ogawa H., and Tsukahara T. Effect of non-glucocorticoid 21-aminosteroid U74006F on experimentation cerebral vasospasm. Surg. Neurol. 31: 190-194. 1989.

Vollrath B., Weir B., and Cook D. Hemoglobin causes release of inositol trisphosphate from vascular smooth muscle. Biochem. Biophys. Res. Commun. 171: 506-511. 1990.

Vollrath B., Weir B., Macdonald R. L., and Cook D. Intracellular mechanisms involved in the responses of cerebrovascular smooth muscle cells to hemoglobin. J. Neurosurg. 80: 261-268. 1994.

Vollrath B., Chan P., Findlay M., and Cook D. Lazaroids and deferoxomine attenuate the intracellular effects of oxyhemoglobin in vascular smooth muscle. Cardiovasc. Res. 30: 619-626. 1995.

Vollrath B., Cook D., Megyesi J., Findlay J., and Ohkuma H. Novel mechanism by which hemoglobin induces constriction of cerebral arteries. Eur. J. Pharmacol. 361: 311-319. 1998.

Walsh M., Horowitz A., Clement-Chomienne O., Andrea J., Allen B., and Morgan K. Protein kinase C mediation of Ca^{2+}-independent contractions of vascular smooth muscle. Biochem. Cell Biol. 74:485-502. 1996.

Wang J., Ohta S., Sakaki S., Araki N., Matsuda S., and Sakanaka M. Changes in the Ca^{2+} ATPase activity in smooth-muscle cell membranes of the canine basilar artery with experimental subarachnoid hemorrhage. J. Neurosurg. 80: 269-275. 1994.

Warnell P. Advanced concepts in the management of cerebral vasospasm associated with aneurysmal subarachnoid hemorrhage. J. Neurosurg. 80: 269-275. 1994.

Way K., Chou E., and King G. Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends in Pharmacol. Sci. 21: 181-187. 2000.

Weir B., Grace M., Hansen J., Rothberg C. Time course of vasospasm in man. J. Neurosurg. 8: 173-178. 1978.

Weir B. The pathophysiology of cerebral vasospasm. Br. J. Neurosurg. 9: 375-391. 1995.

Wellum G., Irvine T., and Zervas N. Cerebral vasoactivity of heme proteins in vitro. Some mechanistic consideration. J. Neurosurg. 56: 777-783. 1982.

Wever R., Oudega B., van Gelder BF., Generation of superoxide radicals during the autooxidation of mammalian oxyhemoglobin. Biochim. Biophys. Acta 302: 475-478. 1973.

Wickman G., Vollrath B. Effects of tamoxifen on oxyhemoglobin-induced cerebral artery vasoconstriction. Eur. J. Pharmacol. 390: 181-184. 2000.

Wilkinson S., Parker P., and Nixon J. Isozyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem. J. 294: 335-337. 1993.

Wiseman H., Cannon M., Arnstein H., and Halliwell B. Mechanism of inhibition of lipid peroxidation by tamoxifen and 4-hydroxytamoxifen introduced into liposomes. Similarity to cholesterol and ergosterol. FEBS lett. 274: 107-110. 1990.

Wiseman H., Cannon M., Arnstein H., and Barlow D. The structural mimicry of membrane sterols by tamoxifen: evidence from cholesterol coefficients and molecular modeling for its action as a membrane antioxidant and anti-cancer agent. Biochim. Biophys. Acta 1138: 197-202. 1992.

Wiseman H., Cannon M., Arnstein H., and Halliwel B. Tamoxifen inhibits lipid peroxidation in cardiac microsomes. Comparison with liver microsomes and potential relevance to the cardiovascular benefits associated with cancer prevention and treatment by tamoxifen. Biochem. Pharmacol. 45: 1852-1855. 1993.

Wiseman H. Tamoxifen: new membrane-mediated mechanisms of action and therapeutic advances. Trends in Pharmacol. Sci. 15: 83-89. 1994.

Yamamoto S., Nishizawa S., Yokoyama T., Ryu H., and Uemura K. Subarachnoid hemorrhage impairs cerebral blood flow response to nitric oxide but not to cyclic GMP in large cerebral arteries. Brain Res. 757: 1-9. 1997.

Yamaura T., Tani E., Maeda Y., Minami N., and Shindo H. Endothelin-1 of canine basilar artery. J. Neurosurg. 76: 99-105. 1992.

Yamaura T., Tani E., Saido T., Suzuki K., Minami N., and Maeda Y. Calpain calpastatin system of canine basilar artery in vasospasm. J. Neurosurg. 79: 537-543. 1993.

Yanagisawa M., Kurihara H., Kimura S., et al. A Novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411-415. 1988.

Yanagisawa M., and Masaki T. Endothelin, a novel endothelium-derived peptide. Pharmacological activities, regulation and possible roles in cardiovascular control. Biochem. Pharmacol. 38: 1877-1883. 1989.

Zervas N., Hori H., and Rosoff C. Experimental inhibition of serotonin by antibiotic: prevention of cerebral vasospasm. J. Neurosurg. 41: 59-62. 1974.

Zervas N., Candia M., Candia G., Kido D., Pessin M., Rosoff C., and Bacon V. Reduced incidence of cerebral ischemia following rupture of intracranial aneurysms. Surg. Neurol. 11: 339-344. 1979.