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EFFECTS OF AMINOGLYCOSIDE ANTIBIOTICS ON INTRACELLULAR PROCESSES IN CEREBRAL VASOSPASM

BY

MOURAD A. NESSIM GERGAWY ©

A thesis submitted to the Faculty of Graduate Studies and Research
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TO HANAA

Abstract

Oxyhemoglobin (OxyHb) and endothelin-1 (ET-1) have been implicated as putative mediators of cerebral vasospasm following rupture of an intracranial aneurysm. However, the possible mechanism by which these agents might induce sustained constriction of cerebral arteries is unclear. Previous work has shown that the contractile effects of OxyHb in cerebral arteries can be prevented or reversed by the aminoglycoside antibiotic, neomycin, an inhibitor of phospholipase C (PLC). We have therefore examined whether related aminoglycoside antibiotics may have similar effects on sustained vasoconstriction produced by the spasmogens implicated in the pathogenesis of cerebral vasospasm. We also attempted to determine the mechanisms by which the aminoglycosides reverse the effects of OxyHb.

The methods used in these studies included contractility experiments and intracellular calcium $[(Ca^{2+})]_i$ measurements using Fura2-AM. Spastic carotid arteries were obtained from the canine model of vasospasm and the presence of vasospasm was confirmed angiographically. All the aminoglycosides produced a concentration dependent relaxation of basilar artery rings preconstricted with either OxyHb or ET-1. The order of potency of the aminoglycosides, against the spasmogenic action of these compounds, was neomycin> gentamicin> streptomycin> kanamycin. This order of potency reflects the number of positive charges each compound possesses; neomycin and gentamicin, which have 6 and 5 ionizable amino groups, respectively, were the most potent ($IC_{50's}$ of about 0.5 mM), while kanamycin and streptomycin which have 4 and 2 amino groups,

respectively, were less effective ($IC_{50's}$ of about 1.5-3 mM). Neomycin inhibited vasoconstriction induced by PGF2α in Ca^{2+} free medium, an observation which is consistent with the reported ability of neomycin to inhibit PLC. The aminoglycosides also relaxed contractions caused by depolarizing concentrations of KCl, a process which is independent of PLC, thus suggesting that the aminoglycosides also inhibit voltage dependent calcium channels (VDCC). Phorbol myristate acetate (PMA) produced a sustained constriction of cerebral arteries, mediated via PKC activation, which was attenuated by all the aminoglycosides. In the studies of $[(Ca^{2+})]_i$ levels, the aminoglycoside antibiotics administered to cerebrovascular smooth muscle cells (CVSM) in the concentrations corresponding to the $IC_{50's}$ determined in contractility experiments, produced approximately 50% inhibition of the $[(Ca^{2+})]_i$ accumulation induced by OxyHb (1μM). Exposure of CVSM cells to the aminoglycosides, added in the presence of OxyHb, for 48 and 72, h resulted in significant inhibition of $[(Ca^{2+})]_i$ level elevated by OxyHb. The present studies provide evidence that in addition to their ability to inhibit PLC, the aminoglycoside antibiotics are effective inhibitors of PKC and intracellular calcium, the key processes thought to be involved in the pathogenesis of cerebral vasospasm. These actions of aminoglycosides seem to be related to the positively charged amino groups, these compounds possess. This implies that the polycationic aminoglycoside antibiotics may be of potential benefit in the management of cerebral vasospasm.

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AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium
CaM	calmodulin
CSF	cerebrospinal fluid
CVSM	cerebrovascular smooth muscle
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
ECE	endothelin converting enzyme
EDRF	endothelium derived relaxing factor
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EPSP	excitatory postsynaptic potential
ET	endothelin
ET_A	endothelin A receptor
ET_B	endothelin B receptor
Fura-2 AM	Fura-2 acetoxymethyl
GMP	guanosine monophosphate
IC_{50}	50% inhibitory concentration
I_{CRAC}	calcium release activated current
Ins (1,4,5) P_3	inositol (1,4,5) trisphosphate
IV	intravenous
kDa	kiloDalton
KCl	potassium chloride

MAP kinase	mitogen activated protein kinase
MetHb	methemoglobin
MLCK	myosine light chain kinase
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
PA	phosphatidic acid
PAH	phosphatidic acid hydrolase
PC	phosphatidylcholine
PGF2α	prostaglandin F2α
PIP_2	phosphatidyl inositol (4,5) bisphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
OxyHb	oxyhemoglobin
SAH	subarachnoid hemorrhage
SEM	standard error of the mean
SH	src homology
SR	sarcoplasmic reticulum
VDCC	voltage dependent calcium channels

CHAPTER ONE

INTRODUCTION

Subarachnoid hemorrhage is a common condition that affects approximately 25000 persons in the United States and Canada each year (Adams, 1992). A major complication of subarachnoid hemorrhage is cerebral vasospasm which was first described by Ecker and Riemenschneider in 1951.

There are two definitions of vasospasm, one angiographic and the other clinical. Angiographic vasospasm is a sustained narrowing of the angiographic column in the major cerebral arteries occurring after subarachnoid hemorrhage, while clinical vasospasm is the syndrome of the ischemic consequences of cerebral artery narrowing (Ecker and Riemenschneider, 1951).

Early clinical manifestations of vasospasm include persistent or increasingly severe headache and decreased level of consciousness. This may be followed by focal neurological signs such as aphasia and paralysis. Also delayed fever beginning a few days after the subarachnoid hemorrhage may be a sign of vasospasm. Cerebral vasospasm follows a characteristic time course, starting 3-4 days after the hemorrhage, reaching a peak at day 7-10 and then tapering after 2 weeks. The time course of clinical vasospasm parallels that of angiographic vasospasm (Adams, 1992 ; Macdonald, 1995 ; Pickard *et al.*, 1992)

The reported incidence of vasospasm depends on the timing and criteria for diagnosis. It is estimated, however, that 60 to 70% of victims of subarachnoid hemorrhage will develop angiographic vasospasm and approximately 30% will experience clinical vasospasm (Kassell *et al.,* 1985). Further, it is believed that for symptomatic vasospasm to develop, the cerebral vessels have to be 50% or more occluded.(Kassell *et al.*, 1985).

The mechanism and pathogenesis of vasospasm have been extensively studied but are not yet completely understood. Many studies have shown that the amount of blood collected in the subarachnoid space is a faithful predictor of the development and severity of the spasm (Adams *et al.,* 1987 *;* Fischer *et al.,* 1980).

There is growing consensus that the release of spasmogens as a result of lysis of erythrocytes trapped in the subarachnoid space is the most important factor in the etiology of vasospasm. Indeed, the time course of erythrocyte lysis coincides with clinical vasospasm (Pickard *et al.*, 1992).

Significant advances have been made in the management of vasospasm that lead to improvement of its outcome. Current treatment of vasospasm includes intra-operative blood removal, hypertensive hypervolemic therapy involving the use of vasoconstrictors and volume expanders, calcium channels blockers and transluminal angioplasty. Many experimental new lines of therapy are continually being evaluated. Among the promising candidates are clot lysis using tissue plasminogen activator, intraarterial papaverine infusion and free radical scavengers such as tirilazad (Macdonald, 1995 ; Warnell, 1996). It should be noted, however, that vasospasm still carries a grave prognosis and 15% of clinical vasospasm patients will either die or have permanent disability (Kassell *et al.*, 1990). Vasospasm is also notoriously resistant to vasodilators and there is a clear need for an effective drug to reverse or prevent the arterial narrowing.

I. PATHOGENESIS OF VASOSPASM

There are two broad hypotheses which have been advanced to explain the pathogenesis of vasospasm. The first hypothesis states that vasospasm is primarily a prolonged and temporarily irreversible smooth muscle contraction that leads to ultrastructural changes in the vessel wall. The second hypothesis is referred to as "the structural hypothesis" which proposes that the structural changes in the vessel wall are themselves the cause of vasospasm (Findlay *et al.*, 1989 and 1991). An early paper by Crompton reported structural changes in vessels taken from patients with angiographically confirmed vasospasm who died more than 3 weeks after subarachnoid hemorrhage (Crompton, 1964). It is difficult to implicate the vasculopathy observed in this study as a cause of

vasospasm because the vessels were taken at a time when clinical vasospasm has resolved, and also it was not clear whether these changes are distinct from atherosclerotic changes expected in these patients. Since this early report many groups studied the structural changes in vessels after subarachnoid hemorrhage using animal models as well as human vessels. Unfortunately the results are conflicting and the question of the importance of structural changes as a cause of vasospasm is unresolved.

Hughes and Schiachi (1978), conducted a histological study on vessels from patients dying after SAH; they reported that early changes were mainly necrosis of the tunica media while late changes were concentric intimal thickening by subendothelial fibrosis. They suggested that these changes are the result of ischemia rather than the cause of vasospasm. Pickard and co-workers (1985) used a canine model of experimental vasospasm and failed to detect ultrastructural changes even in areas of angiographic spasm, using scanning and transmission electron microscopy. Findlay and colleagues (1989) proposed that vasospasm was due to contraction of medial smooth muscle followed by muscular degeneration. They argued against intimal thickening being the initiating event in the development of vasospasm. The observation that histological changes are minimal when spasm is maximal, and that these changes appear when vasospasm is resolving suggests that histological changes are a result rather than a cause of vasospasm (Hughes and Schiachi, 1978). It is becoming accepted that vasospasm is a state of smooth muscle contraction and that changes in the vessel walls reflect a response of the arterial wall to the period of prolonged contraction.

Many spasmogens have been implicated in the pathogenesis of vasospasm and attempts to pin point a single factor in the causation of vasospasm have not been successful. There is a growing consensus that OxyHb and endothelin are the major spasmogens, while other agents such as free

radicals, eicosanoids or disturbance of NO production may also play important roles in the pathogenesis of vasospasm.

1. MAJOR SPASMOGENS INVOLVED IN VASOSPASM

a) OXYHEMOGLOBIN

The incidence and severity of vasospasm correlate with the size and extent of the subarachnoid clot around the vessels, so it is conceivable that substances released from the clot cause the vasospasm (Macdonald and Weir, 1991). The principal pathogenic agent in vasospasm is probably OxyHb which is released from RBC's in the subarachnoid clot. This hypothesis is supported by a number of observations. First, OxyHb has a vasoconstrictive effect on cerebral arteries (Cook *et al.,* 1979 ; Tanishima, 1980 ; Wellum *et al.,* 1982). Second, the time-course of the release of OxyHb from RBC's correlates with the development of vasospasm in patients after SAH (Macdonald and Weir, 1994 ;Okwuasaba *et al.*, 1981 ; Ozaki and Mullen, 1979). Third, OxyHb induces a sustained increase in intracellular calcium level lasting for days and the administration of OxyHb to cerebrovascular smooth muscle cells mimics the morphological changes observed in spastic cerebral arteries (Steel *et al.*, 1990 ; Vollrath *et al.*, 1995). Perhaps the most important evidence was provided by the findings that OxyHb administered *in vivo* produced sustained vasospasm in the monkey model, while methemoglobin and bilirubin were without effect (Macdonald *et al.* 1991). Although OxyHb released from the blood clot may be the pathogenic agent in cerebrovascular spasm, the mechanism by which this agent causes cerebrovascular spasm is unclear. The intracellular mechanisms proposed to explain the vasospastic action of OxyHb include: 1. free radical generation during the oxidation of OxyHb to MetHb and subsequent lipid peroxidation (Steel *et al.*,

1991), 2. stimulation of endothelin secretion (Kasuya *et al.*, 1993), 3. disturbance of endothelial regulation of vascular tone by binding to nitric oxide (Hongo *et al.*, 1988), and 4. imbalance in the synthesis of eicosanoids produced by the ability of OxyHb to increase the levels of prostaglandins with vasospastic activity and to decrease the levels of vasorelaxant prostacyclins (Nosko *et al.*, 1988).

Free radicals

The observation that OxyHb containing ferrous iron, consistently produces vasoconstriction and mimics vasospasm while MetHb, in which the iron is present in the ferric form, does not, suggests that the state of oxidation of iron may play a role in the development of vasospasm. This hypothesis is supported by the finding that iron chelators such as, desferoxamine, inhibit the contractile actions of OxyHb both *in vitro* and *in vivo* (Harada and Mayberg,1992 ;Vollrath *et al.*, 1995). The oxidation of OxyHb to MetHb releases superoxide anion which interacts with water to form hydrogen peroxide, which can react in turn with ferrous iron to produce the highly reactive hydroxyl radical. This free radical has been shown to initiate and propagate lipid peroxidation by the iron-catalyzed Haber-Weiss or Fenton reaction. The mechanism by which hydroxyl radical initiates lipid peroxidation is mediated by abstracting a hydrogen atom from polyunsaturated fatty acids such as arachidonic acid (Gutteridge and Halliwell, 1990 ; Puppo and Halliwell, 1988 ; Wever *et al.,* 1973). The formation of lipid peroxides in the plasma membrane causes impairment of membrane function, changes in the membrane permeability, formation of membrane blebs and eventually cell death (Steel *et al.,* 1990). Although free radicals derived from OxyHb have been implicated in the pathogenesis of cerebral vasospasm, experimental evidence for this proposal is limited. In some studies the xanthine oxidase/ xanthine reaction was used as a source of oxygen radicals. Intracisternal injection of the mixture of these compounds and ferrous iron produced sustained vasoconstriction in an *in vivo* model of vasospasm

(Kamaiyama *et al.*, 1981). Injections of lipid peroxide were also shown to produce vasoconstriction of cerebral arteries in animal models of vasospasm (Kamaiyama *et al.*, 1981 ; Sasaki *et al.*, 1981). Furthermore, the concentration of lipid peroxides in the CSF of patients after SAH was shown to be elevated (Sasaki *et al.*, 1979 and 1980). There is also evidence that OxyHb and/or free radicals administered in vitro to cerebrovascular smooth muscle cells produces morphological changes compatible with those observed in the cerebral arteries of patients after SAH (Steel *et al.*, 1990).

If free radicals generated by OxyHb represent a prominent mechanism involved in the initiation of vasospasm, then catalase, which catalyses the degradation of hydrogen peroxide, or superoxide dismutase involved in the conversion of superoxide anion to hydrogen peroxide, might be expected to ameliorate vasospasm. There is conflicting evidence that these enzymes may exert protective effects. Some studies have shown that SOD and catalase were effective in inhibiting vasospasm (Kamiyama *et al.*, 1981) However, similar studies have failed to demonstrate such protective effects (Macdonald *et al.*, 1992 ; Wellum *et al.*, 1982). Inconsistent reports regarding the effectiveness of these compounds may be due to the inability of these large molecules to reach the site of free radical formation. In support of this hypothesis is the observation that human recombinant SOD which has high penetrating capacity was effective in vivo against vasospasm in a rabbit model when it was directly injected into the subarachnoid cisterns (Shishido *et al.*, 1993).

Evidence that oxygen radicals and lipid peroxides may be involved in the pathogenesis of vasospasm suggest that compounds with antioxidant activity such as aminosteroids might be of potential value in the treatment of vasospasm. 21-aminosteroids are steroid compounds lacking glucocorticiod activity (Braughler *et al.*, 1987). These compounds inhibit lipid peroxidation by scavenging oxygen free radicals. They also inhibit propagation of lipid peroxidation, stabilize the cell membranes and may also chelate ferrous iron (Braughler *et al.*, 1988 ; Braughler and Pregenzer, 1989). The compound

U74006F or tirilazad mesylate, has been shown to ameliorate cerebral vasospasm in animal models of vasospasm (Steinke *et al.*, 1989 ; Vollmer *et al.*, 1989). This success lead to clinical trials which have at least suggested that tirilazad reduces the severity of vasospasm allowing for a more favorable outcome for vasospasm patients (Kassell *et al.*, 1996). The success of tirilazad in ameliorating vasospasm would lend strong support to the hypothesis that free radicals and lipid peroxidation products are involved in the pathogenesis of vasospasm. Another important action of OxyHb is its ability to stimulate expression of endothelin, a powerful spasmogen thought to be involved in the pathogenesis of vasospasm.

b) ENDOTHELIN

Endothelin was discovered by Yanagisawa and his colleagues (1988) as a novel and potent vasoactive peptide released from endothelial cells. Further studies have shown that endothelin is a member of a family of vasoactive peptides which include endothelins 1, 2 and 3 (Inoue *et al.*, 1990). Endothelins are synthesized as preproendothelin which is cleaved by an endopeptidase to big endothelin, a less active peptide than mature endothelin. Big endothelin is then cleaved by an endothelin converting enzyme (ECE) to endothelin. Endothelin is the most potent endogenous vasoconstrictor and is characterized by long action on smooth muscle (Haynes and Webb, 1993 ; Warner, 1993). Two endothelin receptors have been isolated and cloned from mammalian tissues : ET_A and ET_B receptors both coupled to a G-protein. These receptors have different affinity for endothelin peptides; ET_A receptor binds ET-1 and ET-2 peptides with high affinity but ET-3 with low affinity while a non selective ET_B receptor binds all endothelin peptides with equal affinity (Arai *et al.*, 1990 ; Sakurai *et al.*, 1992).

Activation of endothelin receptors is associated with activation of a Gq protein which in turn stimulates PLC activity, Ins (1,4,5)P_3 formation, intracellular Ca^{2+} release and DAG generation. Endothelin is also known to induce Ca^{2+} influx through both receptor and voltage dependent Ca^{2+} channels. Another important property of endothelin is its mitogenic effect in vascular smooth muscle. This effect is thought to be mediated by a src family tyrosine kinase which are involved in the activation of mitogen activated protein kinase (MAPK) and gene expression (Aramore and Nakanishi, 1992 ; Simonson *et al.*,1992 ; Yanagisawa and Masak, 1989)

Since its discovery, endothelin has raised a special interest in relation to vasospasm. Many studies were conducted to explore the involvement of endothelin in cerebral vasospasm. These studies have shown that even nanomolar concentrations of endothelin produce a potent and sustained constriction of isolated cerebral arteries (Ide *et al.*, 1989 ; Saito *et al.*, 1989). Furthermore, intrathecal injection of endothelin induced a sustained vasospasm in animal models of SAH (Asano *et al.*, 1989 ; Kobayashi *et al.*, 1990). Levels of endothelin are elevated in the CSF of patients after SAH and in animal models, although these studies remain controversial (Cosentino and Katusic, 1994 ; Hamman *et al.*, 1993 ; Roux *et al.*, 1995 ; Seifert *et al.*, 1995). It has also been demonstrated that endothelin receptor antagonists may prevent or reverse experimental vasospasm. Numerous studies in which the canine model of vasospasm was used, reported beneficial effects of ET_A and ET_B receptor antagonists (Itoh *et al.*, 1993 and 1994 ; Nieri *et al.*, 1993). Phosphoramidon, an ECE inhibitor, was also shown to be effective in ameliorating vasospasm, thus confirming the involvement of endothelin synthesis in the generation of vasospasm (Matsumara *et al.*, 1991). The observation that OxyHb and/or free radicals derived from OxyHb stimulate endothelin production in both endothelial and smooth muscle cells, provide an important link between these two agents in

the pathogenesis of vasospasm. This action of OxyHb is mediated most likely through the activation of PKC (Kasuya *et al.*, 1993 ; Ohlstein and Storer, 1992).

c) NITRIC OXIDE (NO)

The impairment of endothelium-dependent vasodilation has been proposed to be involved in the pathogenesis of cerebral vasospasm (Hongo *et al.*, 1988 ; Kim *et al.*, 1989). Endothelium is a source of EDRF (endothelium derived relaxing factor) recently identified as nitric oxide (NO) (Furchgott *et al.*, 1980 ; Palmer *et al.*, 1988).

NO is formed from L-arginine by the action of two different types of NO synthases. One type is a constitutive, calmodulin dependent NOS which is present in the endothelium and the neuronal tissue (Moncada *et al.*, 1991 ; Palmer *et al.*, 1988). This enzyme is activated by calcium which binds to calmodulin, forming a complex that is an important cofactor for enzyme activity. In vascular endothelium this calcium originates from the action of receptor agonists such as acetylcholine and bradykinin, each of which generate Ins (1,4,5) P_3 which in turn releases intracellular calcium from SR. NO relaxes blood vessels by binding to iron in the heme at the active site of guanylyl cyclase in smooth muscle, thereby activating the enzyme and generating cyclic GMP. NO can also exert direct effect on smooth muscle via calcium dependent K^+ channels (Butler *et al.*, 1995 ; Hunley *et al.*, 1995 ; Schmidt *et al.*, 1993). The other type of NOS is an inducible NOS which is expressed in many cells including smooth muscle cells, after stimulation with immunologic or inflammatory stimuli (Busse and Mulsch 1990 ; Forstermann *et al.*, 1994).

The most characteristic property of NO in terms of its biological activity is the interaction with iron which is responsible for the activation of guanylyl cyclase and for the binding to other hemoproteins (Schmidt *et al.*, 1993).

Hemoglobin is known to bind and thus inactivate NO (Cocks and Angus, 1985) As mentioned earlier it is well documented that endothelium-dependent relaxation is diminished in cerebral arteries from animal models of subarachnoid hemorrhage (Kim *et al.,* 1992). There is also evidence that injections of OxyHb *in vivo* produced vasoconstriction of cerebral arteries and reversed the vasodilatory effect of acetylcholine, thus suggesting that endothelium-mediated vasodilation was impaired and that this effect may contribute to the OxyHb-mediated effects on blood vessels (Kim *et al.*, 1989 and 1992 ; Nakagomi *et al.*, 1987).

In addition to its vasodilatory effect NO in high, non physiological concentrations may also be detrimental to blood vessels. The reaction of superoxide anion with NO generates peroxynitrite ion which decomposes to nitrogen dioxide and the highly toxic hydroxyl radical. It is possible that superoxide anions produced by autooxidation of OxyHb play a role in these reactions (Beckman *et al.*, 1990 ; Bondy and Naderi, 1994).

d) EICOSANOIDS

An imbalance in the production of eicosanoids has been reported in cerebral vessels from animal models of vasospasm. In these studies, an increase in the level of contractile prostaglandins, including PGF2α, as well as a decrease in the synthesis of vasodilatory prostacyclins were reported (Macdonald and Weir, 1990 ; Maeda *et al.*, 1981 ; Nosko *et al.,* 1988). OxyHb was shown to decrease the concentrations of PGI2 (Tokoro, 1984) and to stimulate the synthesis of prostaglandins in blood vessels (Toda, 1990). It is thought that these actions of OxyHb are mediated via lipid peroxidation and activation of PLA_2 with subsequent release of the products of the arachidonic acid cascade (Macdonald and Weir, 1990). If eicosanoids play a major role in the pathogenesis of vasospasm then cyclooxygenase inhibitors should reverse or ameliorate the spasm. However, the studies of the effects of these inhibitors on

vasospasm are inconclusive. Aspirin and indomethacin, inhibitors of the cyclooxygenase pathway, were shown to have very little or no effect on experimental or human vasospasm (Fukumori *et al.*, 1983 ; Macdonald and Weir, 1990). Some studies showed beneficial effects of thromboxane inhibitors (Fukumori *et al.*, 1984). However, these agents may represent some potential hazard with respect to haemostasis, and therefore may be of limited clinical use.

II. MECHANISM OF SMOOTH MUSCLE CONTRACTION

Stimulation of smooth muscle with vasoconstrictors results in a biphasic cellular response. The response is initiated by an increase in the level of calcium released from intracellular stores, and subsequent influx of extracellular calcium. Intracellular calcium $[Ca^{2+}]_i$ binds to calmodulin (CaM), a calcium binding protein, in a ratio 4:1 thus leading to sequential conformational changes in CaM, and exposure of the site of interaction with target proteins (Klee, 1980). Ca^{2+} - CaM complex binds to myosin light chain kinase (MLCK) and activates this enzyme by relieving its autoinhibition (Kemp and Pearson, 1991). The activated MLCK phosphorylates specific serine residues in the two 20 KDa light chain subunits of myosin (Adelstein and Klee, 1981). The phosphorylated myosin heads then interact with actin thin filaments, thus leading to smooth muscle shortening and force generation. The mechanisms involved in smooth muscle contraction are shown in Fig. 1.

There are at least three mechanisms involved in the increase of intracellular calcium levels in smooth muscle. The first is the release of $[Ca^{2+}]_i$ initiated by $(Ins1,4,5)P_3$ derived from hydrolysis of inositol phospholipids, the second is the release of $[Ca^{2+}]_i$ from the ryanodine receptor-operated pool, and the third is Ca^{2+} entry through receptor or second messenger regulated Ca^{2+}

channels and/or voltage operated Ca^{2+} channels (Fasolato *et al.*, 1994). In many ways these mechanisms affect one another and the relative contribution of these mechanisms depend on vasoactive agonists and on the type of smooth muscle (Aaronson, 1994 ; Alborch *et al.*, 1995 ; Missiaen *et al.*, 1992 ; Somlyo and Somlyo, 1994).

The involvement of the voltage dependent, L-type calcium channels in vasospasm attracted attention as early as 1979, when nifedipine was reported to prevent vasospasm in a canine model (Allen and Bahr, 1979). This pioneering study was followed by numerous studies testing different calcium channel blockers of the dihydropyridine group on cerebral vasospasm. A clinical trial conducted by Allan and co-workers (1983) demonstrated that nimodipine significantly reduced the severity of ischemic neurologic deficit in patients with SAH. It has been shown later that the effects of this compound on ischemic deficit were not due to the dilation of the cerebral vessels. Studies using primate models of vasospasm have consistently failed to show that calcium channel blockers prevent vasospasm (Espinosa *et al.*, 1984 ; Nosko *et al.*, 1985). Currently, it seems clear that voltage gated calcium channels do not play a critical role in the initiation or maintenance of cerebral vasospasm, and the beneficial effects of nimodipine in vasospasm after SAH are mediated through a mechanism distinct from vasorelaxation (Pickard *et al.*, 1989). Calcium channel blockers are now part of the standard treatment of vasospasm due to their documented neuroprotective effects (Warnall, 1996).

The principal mechanism of intracellular calcium elevation and smooth muscle contraction in response to receptor agonists is known as pharmacomechanical coupling. Many agonists such as endothelin, PGF2α, and noradrenaline bind to G-protein coupled receptors in the plasma membrane leading to phospholipase C activation, phosphatidyl inositol (4,5) bisphosphate (PIP_2) hydrolysis and subsequent release of two second messengers, Ins (1,4,5) P_3 and diacylglycerol (DAG). DAG binds to and activates PKC (Nishizuka, 1992)

and this process will be discussed later in this chapter. IP_3 interacts with a specific receptor on the sarcoplasmic reticulum (SR) and induces intracellular calcium release which is followed by calcium influx from the extracellular space (Chadwick *et al.*, 1990 ; Ferris and Snyder, 1992). The mechanism by which Ins (1,4,5) P_3 stimulates Ca^{2+} influx is unclear but is thought that Ins (1,4,5) P_3 may stimulate calcium entry indirectly through a capacitative calcium entry mechanism as proposed by Putney (1986). The influx of extracellular calcium seems to be regulated by the calcium content of Ins (1,4,5) P_3 -dependent Ca^{2+} pool. Thus, although the PIP_2-linked receptors release calcium only transiently, intracellular Ca^{2+} increases are prolonged substantially by the capacitative entry mechanism. The putative ion channel involved in this mechanism is referred to as I_{CRAC} (Ca^{2+} release-activated current) (Hoth and Penner, 1992).

Three major types of PIP_2 -PLC β, γ, and δ have been identified on the basis of sequences homology, with at least nine different subtypes : 3 for β, 2 for γ and 4 for δ (Rhee *et al.*, 1989). PIP_2 -PLC β type is activated by receptor stimulation at low physiological concentrations of calcium (Berridge and Irvine, 1989). These enzymes are linked to the Gq-protein family. PIP_2 -PLC β can also be activated by the βγ subunit of the Gi protein. The mechanism of activation of PLC γ involves activation of tyrosine kinase (Rhee and Choi, 1992 a). The phosphorylated tyrosine residues bind to src homology domains (SH_2 and SH_3) of PLC γ thus leading to PLC γ activation (Rhee and Choi, 1992 b). Little is known about the mechanism of activation of PLC δ.

The expression of PLC β, PLC γ and PLC δ isoforms and the level of Ins (1,4,5) P_3 , are elevated in the CSF of patients after SAH, which suggests that these enzymes may be involved in cerebral vasospasm (Nakashima *et al.*, 1993). This suggestion is confirmed by the observations that OxyHb-mediated stimulation of Ins (1,4,5) P_3 formation, intracellular calcium release and

vasoconstriction are inhibited by neomycin, a well known inhibitor of PLC (Vollrath *et al.*, 1990).

It is accepted that stimulation of smooth muscle results in biphasic contractile and cellular responses in which a transient phase is followed by a sustained prolonged phase. In contrast to the initial phase, which is activated by calcium increase, the sustained phase is characterized by DAG accumulation and by prolonged activation of PKC (Rasmussen *et al.*, 1987). Agonist mediated stimulation of smooth muscle usually results in a rapid, transient rise in DAG which is followed by a prolonged DAG accumulation and activation of PKC. Transient rise in DAG is caused by hydrolysis of PIP_2 and coincides in time with an increase in Ins (1,4,5) P_3 (Berridge, 1987 ; Berridge, 1993 ; Lee and Severson, 1994). The sustained elevation of DAG is generally believed to result from the hydrolysis of phosphatidylcholine (PC) because the fatty acid composition of DAG formed in the sustained phase of the response corresponds to that of PC (Jarpe *et al.*, 1994 ; Lassegue *et al.*, 1993 ; Nishizuka, 1995). DAG can be generated from PC by one of two mechanisms. The first of them is hydrolysis catalyzed by PC-specific PLC, which is not very well documented, and the second is the concerted action of phospholipase D (PLD), which generates phosphatidic acid (PA), and phosphatidic acid hydrolase (PAH) which in turn metabolizes PA to DAG (Nishizuka, 1995).

DAG and phorbol esters, powerful activators of PKC, bind to a specific site in the regulatory domain of PKC thus leading to its activation. The binding site for DAG and phorbol ester comprises two tandem repeats of a cysteine rich zinc finger motifs (Burns and Bell, 1991 ; Gschwendt *et al.*, 1991). At least eleven isoforms of PKC have been identified in mammalian tissues. They fall into three groups: 1.) the first group is the "classical" PKC's comprising α, βI, βII and γ isoforms which require DAG or phorbol ester, Ca^{2+} and phsphatidylserine (PS); 2.) the second group is the "novel" PKC's (δ, ε, η and θ) which require DAG or phorbol ester and PS; 3.) and third group includes "atypical" isoenzymes (ζ and

λ) which require only PS (Nishizuka, 1995). Cerebral arteries have been shown to contain α, ε and ζ isoforms (Matsui *et al.*, 1993).

Numerous studies have provided evidence supporting a role for DAG and PKC in the pathogenesis of cerebral vasospasm. Recognition of the involvement of PKC in vasospasm stemmed from the observation that phorbol esters which directly activate PKC produce a sustained constriction of isolated cerebral arteries (Sugawa *et al.*, 1991). This was followed by the observations that intracisternal injections of phorbol esters produced vasospasm in an *in vivo* canine model (Sako *et al.,* 1993). The content of the endogenous PKC activator, DAG, was reported to increase, over a long period of time, in cerebral arteries of the canine model of vasospasm (Matsui *et al.*, 1991). Inhibitors of PKC such as H7 and staurosporin were shown to ameliorate vasospasm in an *in vivo* animal model (Matsui *et al.*, 1991). The activity of PKC is increased in cerebral arteries in canine experimental vasospasm (Nishizawa *et al.*, 1992 ; Yokota *et al.*, 1995).

The mechanism by which PKC activation might produce vasospasm is unclear, but an important aspect of its action may be the phosphorylation of the actin thin filament proteins calponin and caldesmon and subsequent disinhibition of the myosin interaction with actin (Walsh, 1993). Calponin is a CaM-binding, troponin-like protein present only in smooth muscle (Takahashi and Nadal Ginard, 1991). Calponin acts by inhibiting actin-myosin interaction. Phosphorylation of calponin by PKC prevents the interaction between calponin and actin thus leading to the relief of inhibition of actomyosin-ATP-ase and subsequent constriction (Winder and Walsh, 1990). Caldesmon is also an actin binding protein which is thought to act by decreasing the affinity of phosphorylated myosin light chains to actin. It is present in smooth muscle as well as non-muscle tissue (Marston and Redwood, 1991). Caldesmon is phosphorylated in vitro and in vivo by MAP kinase which is a substrate for PKC. PKC phosphorylates raf kinase, an important component in the MAP kinase pathway, which indirectly activates MAP kinase and hence phosphorylation of

caldesmon (Adam and Hathaway, 1993 ; Strugill and Wu, 1991). Phosphorylation of caldesmon by these kinases reduces its ability to inhibit the affinity of phosphorylated myosin to actin and this results in smooth muscle contraction (Walsh, 1994).

The involvement of calponin and caldesmon in the pathogenesis of vasospasm has been examined using immunoblotting techniques. It has been shown that the immunoreactivity of caldesmon and calponin were significantly decreased in animal models of vasospasm thus suggesting that proteolysis of these smooth muscle regulatory proteins may occur after SAH (Doi *et al.*, 1993 ; Takenaka *et al.*, 1993). Proteolysis may also play a role in a sustained activation of PKC in vasospasm. This process may be mediated by the calcium dependent protease calpain. Calpain is activated by high levels of intracellular calcium. Activated calpain cleaves PKC, separating the catalytic and regulatory subunit of the enzyme. Removal of the inhibitory constraint provided by the regulatory subunit leads to uncontrolled activation of PKC (Nishizuka, 1992). There is evidence that calpain levels are increased in vasospastic arteries (Minami *et al.*, 1992). This finding, and the observation that the level of calpastatin, an endogenous inhibitor of calpain, is reduced in vasospasm, suggests that calpain-mediated proteolysis may also be involved in the pathogenesis of vasospasm (Yamaura *et al.*, 1993).

As mentioned earlier, there is a sustained elevation in intracellular calcium in vasospasm as well as in cerebrovascular smooth muscle cells exposed to OxyHb for several days (Takanashi *et al.*, 1992 ; Vollrath *et al.*, 1994). There is evidence that an elevation in calcium activates calpain which in turn produces proteolytic cleavage of smooth muscle regulatory proteins (Yamaura *et al.*, 1993).

The sustained elevation of intracellular calcium observed after administration of OxyHb suggests that the mechanisms involved in the maintenance of calcium homeostasis may be impaired. One such mechanism is

the Ca^{2+}-Mg^{2+} ATPase, a calcium pump present in plasma membrane and involved in calcium extrusion (Wang *et al.*, 1994). There is evidence that the function of this pump is impaired in vasospasm most likely by the action of calpain. Long term inhibition of this Ca^{2+} pump was observed in canine basilar artery from an animal model of vasospasm. Furthermore, this calcium pump inhibition was shown to be mediated by free radicals and lipid peroxides in erythrocytes. This inhibition was partially reversed after administration of the aminosteroid U-89843D with antioxidant properties (Rohn *et al.*, 1996). These observations taken together may provide an explanation for the accumulation of intracellular calcium observed after administration of OxyHb.

If the sustained elevation of intracellular calcium contributes to persistent smooth muscle constriction observed in vasospasm, then intracellular calcium antagonists would be expected to reduce vasospasm. Indeed, there is evidence that an intracellular calcium antagonist HA 1077, also referred to as AT877, ameliorates vasospasm in a canine model (Shibuya *et al.*, 1988 ; Takayasu *et al.*, 1986). A large multicenter clinical trial has demonstrated that treatment with AT 877 resulted in a significant reduction of angiographic vasospasm (Shibuya *et al.*, 1992). This provides additional evidence for the role of sustained calcium elevation in the pathogenesis of vasospasm.

From the above discussion, it is clear that two key intracellular processes leading to vasospasm involve, 1) sustained elevation of intracellular calcium and 2) the activation of both PLC and PKC. Since neomycin has been shown to be a PLC and calcium antagonist, it was used in our laboratory in previous studies to investigate the involvement of these processes in vasospasm. Evidence from our laboratory as well as from the literature (Hagiwara *et al.*, 1988 ; Goodman *et al.*, 1974) suggests that neomycin has multiple actions, some of which may be relevant to vasospasm. Also actions of neomycin are related to its polycationic nature and are likely to be shared by aminoglycosides with similar structural characteristics. Therefore, the aim of these studies has been to investigate the

effects of aminoglycosides as polycationic compounds on cellular processes, mediated by OxyHb and endothelin possibly involved in the pathogenesis of vasospasm.

III. AMINOGLYCOSIDES

Aminoglycoside antibiotics constitute a group of antibiotics known to be effective against gram negative bacilli. Structurally, the aminoglycosides are similar. They have an aminocyclitol moiety linked glycosidically to two or more amino sugars. This aminocyclitol or hexose nucleus is usually located in a central position, and is either 2-deoxystreptamine (present in most aminoglycosides) or streptidine (present in streptomycin) (Lortholary *et al.*, 1995).

Aminoglycosides are highly polar polycationic structures at physiological pH. The number of positive charges carried by these compounds is proportional to the number of their ionizable amino groups (Hagiwara *et al.*, 1988 ; Josepovitz *et al.*, 1982). This polycationic property of aminoglycosides seems to be responsible for their ability to interact with anionic molecules in the plasma membranes. The best studied processes include interactions of the protonated groups of neomycin with the phosphate head groups of acidic phospholipids such as phosphoinositides, phosphatidic acid and phosphatidyl serine (Marche *et al.*, 1983 ; Sastrasinh *et al.*, 1982).

1. EFFECTS OF NEOMYCIN ON PHOSPHOLIPASE C ACTIVITY

The ability of neomycin to interact with phosphoinositides has been utilized in studies of the involvement of phosphoinositide hydrolysis in biological reactions (Schibeci and Schacht, 1977). An apparently important consequence of the interactions of this compound with phosphoinositides is the inhibition of

phosphoinositide hydrolysis, Ins (1,4,5) P_3 formation and intracellular calcium release from SR. These effects are independent of the bactericidal actions of neomycin, and are shared by other aminoglycosides as well as endogenous polyamines including spermine and spermidine (Marche *et al.*, 1983 ; Schacht, 1976). There seems to be a correlation between the inhibitory effects of the individual aminoglycosides and the number of their positive charges. Neomycin and gentamicin which have 6 and 5 amino groups, respectively, were shown to be more effective inhibitors of phosophoinositide hydrolysis and Ins (1,4,5)P_3 formation than kanamycin with 4 positive charges (Lipsky and Lietman, 1982 ; Marche *et al.*, 1983 ;Sastrasinih *et al.*, 1982). The presence of amino groups in naturally occurring, aliphatic polyamines support this observation and explains why the order of potency of inhibitory effects of these compounds on PLC activity is spermine (4 positive charges) > spermidine (3 positive charged) and > putrescine (2 positive charges) (Schacht, 1976). Inhibitory effects of neomycin and related aminoglycosides on PLC activity have been reported by many authors in a variety of tissues and cells including smooth muscle (Carney *et al.*, 1985 ; Hostetler and Hall, 1982 ; Vollrath *et al.*, 1990). Goding and Vink (1994) examined the protective effects of neomycin on the central nervous system following injury. The authors used phosphorus magnetic resonance spectroscopy and motor function tests to assess the degree of permanent damage induced by injury. The group of rats that received IV neomycin showed a significantly better neurological outcome than the untreated group. This beneficial effect was attributed to the ability of neomycin to inhibit PLC.

It has also been reported that, in addition to the interaction with phosphoinositides, neomycin binds Ins (1,4,5) P_3 and ATP. This action of neomycin was proposed to account for its ability to inhibit intracellular calcium release mediated by Ins (1,4,5) P_3 (Prentki *et al.*, 1986). However, other authors have shown that neomycin does not interact with Ins (1,4,5) P_3 and ATP in intact cells (Lang *et al.*, 1977 ; Tysnes *et al.*, 1987). These latter observations support

the notion that neomycin acts at the level of the plasma membrane and does not permeate into the cytoplasm, which is not surprising in light of the highly polar nature of this agent. Earlier studies in this laboratory have shown that neomycin was an effective inhibitor of Ins (1,4,5) P_3 formation and smooth muscle contraction initiated by OxyHb (Vollrath *et al.*, 1990). These effects were probably mediated by direct interaction of neomycin with negatively charged inositol phospholipids in the plasma membrane, an action which is thought to prevent the enzymatic cleavage of phosphoinositides by PLC. However, it has also been shown that neomycin inhibits contraction produced by OxyHb at a time when the level of Ins (1,4,5) P_3 has returned to the control value (Vollrath *et al.*, 1990). Furthermore, neomycin also diminished the pharmacological responses to potassium chloride which is believed to produce contraction by stimulating Ca^{2+} entry via voltage dependent calcium channels (Vollrath *et al.*, 1990).

These observations suggest that in addition to inhibition of inositol phospholipid hydrolysis neomycin exerts some other actions which contribute to its inhibitory influence on smooth muscle constriction. It is possible that one such action involves calcium entry.

2. EFFECTS OF NEOMYCIN ON CALCIUM INFLUX

Neomycin and related aminoglycosides have been reported to inhibit plasma membrane calcium channels, K^+ and Na^+ channels and to inhibit neuromuscular transmission. (Canzoniero *et al.*, 1993 ; Diecke *et al.*, 1971 ; Dretchen *et al.*, 1972). In rat brain slices, neomycin inhibited potassium-induced norepinephrine release and this inhibition was concentration dependent. This study has also provided evidence that in addition to inhibition of the L-type calcium channels, neomycin can inhibit N-type calcium channels (Keith *et al.*, 1993). Using electrophysiological techniques, Duarte and co-workers (1993) have shown that high milimolar concentrations of neomycin preferentially

blocked the dihydropyridine-insensitive calcium influx in chromaffin cells. Antagonistic interactions between aminoglycoside antibiotics and calcium have also been recognized in the myocardium, autonomic ganglia, kidney neuromuscular junction and smooth muscle (Adams *et al.*, 1974 ; Swain *et al.*, 1956 ; Wolf and Wigton, 1971). In peripheral smooth muscle, neomycin and other aminoglycosides have been reported to decrease $^{45}Ca^{2+}$ uptake and to reduce contractility of isolated preparations (Adams *et al.*, 1974 ; Goodman *et al.*, 1974). Furthermore, preincubation with neomycin consistently inhibited peripheral smooth muscle contractions elicited by administration of Ca^{2+} to organ baths containing a Ca^{2+} free solution, thus indicating that Ca^{2+} entry was inhibited by this aminoglycoside (Adams and Goodman, 1975). Neomycin and gentamicin have also been shown to stimulate Ca^{2+} efflux in blood vessels, although the mechanism involved in this has not been elucidated (Goodman *et al.*, 1974). More recently, neomycin was shown to reduce both vasoconstriction of cerebral arteries in response to OxyHb and the sustained intracellular calcium accumulation observed after exposure of cerebrovascular smooth muscle cells to OxyHb over long time period, suggesting that, in addition to the inhibition of Ins (1,4,5) P_3 synthesis, this compound may also inhibit Ca^{2+} fluxes (Vollrath *et al.*, 1994). In addition to the action in smooth muscle, neomycin and related aminoglycosides, including gentamicin, clindamycin, kanamycin, tobramycin and streptomycin, were shown to inhibit intracellular calcium release from isolated SR in skeletal muscle, a system in which Ins (1,4,5) P_3 is ineffective as a second messenger, thus suggesting that the inhibition of calcium release by these agents is mediated by mechanisms unrelated to the effects on PLC activity (Palade, 1987). Similar comparative studies were performed with endogenously occurring polyamines, spermine, spermidine and their precursor putrescine which, in common with aminoglycosides, possess several amino groups. These studies have suggested that the effectiveness of polyamines, including aminoglycosides and endogenous polyamines, in inhibiting calcium induced a

calcium release mechanism was related to the number of positive charges in the structure of these compounds (Palade, 1987).

3. EFFECTS OF AMINOGLYCOSIDES ON PKC ACTIVITY

Neomycin and several other aminoglycoside antibiotics are effective antagonists of PKC isolated from kidney epithelial cells and brain (Hagiwara *et al.*, 1988). That this inhibition was specific has been demonstrated by the finding that cyclic AMP-dependent protein kinase and myosin light chain kinase activities were not affected by these agents. The inhibition of PKC activity was attributed to the polycationic nature of the aminoglycosides. Two observations suggested that the inhibitory effects of aminoglycosides on PKC are due to the positive charges on aminoglycoside molecules. First, an increase in pH was associated with a decrease in the ability of aminoglycosides to inhibit PKC activity. Second, the rank order of aminoglycoside potency in inhibiting the activity of this enzyme correlated well with the number of amino groups in each compound, thus confirming the proposal that the activity of aminoglycosides is related to their structure(Hagiwara *et al.*, 1988). The structurally related endogenous polyamines, spermine and spermidine have also been shown to inhibit PKC activity (Moruzzi *et al.*, 1987). The rank order of potency of these compounds in inhibiting PKC activity was correlated with the number of positive charges.

4. OTHER ACTIONS OF AMINOGLYCOSIDES

Both aminoglycosides and endogenous polyamines have the ability to modulate the functions of the NMDA (N-methyl-D-Aspartate) receptor. This finding is important because it is known that ischemic brain injury can produce an overstimulation of NMDA receptors leading to excessive Ca^{2+} entry, neuronal cell death and neurological damage (Johnson, 1996). Although the role of

polyamines, in relation to brain damage, and the mechanism of action of these agents is still unclear, it is known that the synthesis of these agents is increased after neurological insult. It has also been shown that polyamines may bind to the specific sites in the receptor and hence potentiate the action mediated by NMDA (Nussenzveig *et al.*, 1991). On the other hand, the polyamines have been proposed to act by an endogenous, protective mechanism by limiting calcium toxicity (Johnson, 1996). Furthermore, milimolar concentrations of spermine have been shown to depress the EPSP (excitatory post synaptic potential) (Di Scenna *et al.*, 1994). Endogenous polyamines have also been shown to affect plasma membrane function when administered extracellularly, via interaction with acidic phospholipids such as phosphoinositides, phosphatidic acid and phosphatidylserine. Furthermore, they were shown to inhibit phospholipases, PKC and to affect calcium homeostasis and mitochondrial function (Schuber, 1989). An important property of polyamines is their ability to protect membranes against damage by free radicals and lipid peroxides. Spermine was shown to provide protection against lipid peroxidation-induced by fatty acids in human breast cancer cells (Chapman and Wallace, 1994). Moreover, in permeabilized human neutrophils exposed to free radical generating agents, spermine downregulated superoxide generation (Ogata *et al.*, 1992). This effect was not observed in intact cells stimulated with spermine, thus indicating that, similar to aminoglycoside antibiotics, these highly charged molecules do not have the ability to cross plasma membranes. The actions exerted by these compounds may vary depending on the method of administration and on the site of action.

It is clear from the preceding discussion that the polycationic compounds, aminoglycosides and endogenous polyamines, have more than one mechanism of action, some of which can be relevant to vasospasm. Agents with such a multiplicity of actions would be suitable to investigate vasospasm, which itself is a multifactorial condition involving many intracellular mechanisms.

The effects of aminoglycosides in relation to cerebral vasospasm was first investigated by Zervas *et al.* (1974), more than two decades ago. It should be pointed out, however, that signal transduction processes and the intracellular mechanisms involved in vasospasm at that time were unknown. The investigators used oral kanamycin together with reserpine to reduce the blood levels of serotonin, a vasoactive amine thought, at that time, to cause vasospasm. This treatment was shown to produce a significant improvement in angiographic vasospasm in a monkey model of cerebral vasospasm. These encouraging results led to a randomized multicenter clinical trial for patients with vasospasm (Zervas *et al.*, 1979). Success in vasospasm amelioration was reported. This results were considered to be sufficient to justify that randomization of patients be terminated and all patients with vasospasm in the participating centers were offered this treatment. However, further studies using a monkey model of vasospasm, cast some doubts on the efficacy of the oral kanamycin, which is absorbed rather poorly from the gastrointestinal tract (Noseworthy *et al.*, 1984).

IV. RESEARCH HYPOTHESIS

This project is based on the hypothesis that both OxyHb and ET-1 stimulate the key processes involved in the development of cerebrovascular spasm and that the aminoglycoside antibiotics are effective inhibitors of these processes.

RESEARCH OBJECTIVES

The specific objectives of these studies are twofold. The first is to determine the relative potency of the various aminoglycosides for their ability to inhibit contractile responses of cerebral arteries as well as the attenuation of intracellular calcium elevation in cultured cerebrovascular smooth muscle cells. The second specific objective is to outline the cellular mechanisms of the observed effects. It is proposed that aminoglycoside antibiotics may attenuate or reverse the cellular actions of OxyHb and ET-1 by a number of mechanisms, such as inhibiting PLC and PKC, attenuating calcium influx or promoting calcium efflux and interfering with lipid peroxidation.

The specific questions which were addressed in these studies include:

Which aminoglycoside antibiotic is the most effective vasorelaxant of contractions produced with major spasmogens implicated in vasospasm, and what is the critical structure required to achieve these effects?

What effect do the aminoglycosides have on the sustained contractions induced with phorbol esters, known activators of PKC?

Which aminoglycoside antibiotics are the effective inhibitors of intracellular calcium levels elevated with OxyHb? What is the time-course of these effects?

Additional questions addressed include:

Do the aminoglycoside antibiotics have a vasorelaxant effect on blood vessels after spasm induced in vivo with blood?

Do the aminoglycosides have a synergistic or additive effect with the subeffective doses of papaverine-induced vasorelaxation?

CHAPTER TWO

MATERIALS AND METHODS

Two main approaches were used in this study, contractility studies and intracellular calcium measurements.

I. CONTRACTILITY EXPERIMENTS

Mongrel dogs weighing 16 to 24 kilograms were used. The experiments were done with strict adherence to the standards of the Canadian Council on animal care. The animals were sacrificed with an overdose of sodium pentobarbital (30 mg/Kg), then the brain was dissected and removed within 5 minutes after sacrifice of the animal. The brain was immediately placed in Krebs-Henseleit solution (in mM ; NaCl 118, KCl 5, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, CaCl 2.5, $NaHCO_3$ 25 and Dextrose 11) aerated with O_2 95% and 5% CO_2. The basilar artery was dissected from the brain stem and cut into 3mm segments using dissecting microscope while immersed in aerated Krebs-Henseleit solution. Basilar artery rings were mounted by using 2 stainless steel hooks in organ bath 10 ml working volume containing Krebs-Hensleit solution bubbled with O_2 95% and CO_2 5% at a temperature of 37. Isometric tension was measured using a force displacement transducer (Grass FT. 03) connected to a polygraph (Grass model 7D). The preparations were allowed to equilibrate under a resting tension of 1 gm for 1 hour before drugs were applied. The viability of the preparations was tested with 60 mM KCl, at the beginning of experiments. Sodium sulfate (15mM) was used as a vehicle control for aminoglycosides and was shown to be devoid of any effect.

1. EXPERIMENTAL PROTOCOLS

a) CONCENTRATION EFFECT CURVES FOR AMINOGLYCOSIDE ANTIBIOTICS AGAINST DIFFERENT AGONISTS

The basilar artery rings were preconstricted with agonists, then vasorelaxant studies were conducted. In these studies, concentration dependent responses to aminoglycosides were obtained. The agonists used to constrict the vessels were, OxyHb (10μM), ET-1 (0.5 nM), PGF2α (1μM), KCl (60mM) and phorbol myristate acetate (160 nM). When OxyHb was used, 5μL antifoam-B was added to the organ bath to prevent foam formation by OxyHb. The aminoglycosides used were : neomycin, streptomycin, kanamycin and gentamicin. The concentrations of aminoglycosides used were 0.01-5mM. The tonic phase of contraction was considered as 100% of constriction and the relaxation by papaverine hydrochloride in the concentration of 0.5mM, as maximal relaxation (0% constriction).The preparations were washed to basal tension and left for 20 minutes between drug applications.

The means and standard errors of the mean for $IC_{50's}$ of the aminoglycosides against different agonists, were calculated from individual concentration effect curves of each aminoglycoside against each agonist.

b) TIME COURSE OF THE EFFECTS OF AMINOGLYCOSIDES

Basilar artery rings were constricted with OxyHb (10μM) and the aminoglycosides were added to different arterial preparations, in which tonic contraction had developed, in a concentration of 5mM and the relaxing effect followed for 3 hours. The effects of aminoglycosides were compared to parallel controls where OxyHb alone was added.

c) EFFECT OF NEOMYCIN IN CALCIUM FREE MEDIUM

Basilar artery rings were equilibrated as described above then the Krebs-Henseleit solution was replaced by calcium free Krebs-Hensleit containing 2 mM of EGTA . Calcium depletion was confirmed by lack of response to 60 mM KCl. The vessels were preconstricted with PGF2α (10μM) and neomycin was added on the top of tension.

d) COMBINED EFFECT OF NEOMYCIN AND PAPAVERINE

Basilar artery rings were preconstricted with PGF2α (1μM), then either papaverine (1μM) or neomycin (1mM) or a combination of both were added to the organ bath and the relaxation of the arterial preparations was then measured.

e) EFFECT OF ENDOTHELIUM REMOVAL ON THE ACTIONS OF NEOMYCIN

In order to examine whether or not the effects of aminoglycosides are dependent on the presence of endothelium, the endothelial cell layer was removed mechanically by gentle scraping, and the arterial preparations were suspended in the usual way. The vessels were preconstricted with either OxyHb (10μM) or PGF2α (1μM). Bradykinin ($10^{-12}$to 10^{-7}M) was then added and the absence of endothelium was confirmed by the lack of relaxing effect of bradykinin. The relaxing effect of Neomycin (5mM) was then examined.

f) EXPERIMENTS USING VASOSPASTIC AND NORMAL CAROTID ARTERIES

Mongrel dogs were also used in this study. Vasospasm was induced by incubating autologous blood around the arteries as previously described in previously (Megyesi et al. in press).Briefly, the animals were anesthetized with sodium pentobarbital (0.5mg /Kg) then intubated and allowed to breathe room air. Prior to SAH a baseline angiogram was obtained. Anaesthesia was maintained by intravenous boluses of pentobarbital as necessary. A midline cervical incision was made and cervical internal carotid artery exposed and punctured with a 26 gauge angiocatheter for angiography. Six cm of both internal carotid arteries were dissected free of adjacent tissues. Ten ml of autologous blood was incubated around the ICA within a 5 cm silastic tube of 1 cm inner diameter. On day 7 the cervical incision was reopened and angiography repeated before the animal was sacrificed with an overdose of pentobarbital (30 mg/kg).The carotid arteries were then removed for pharmacological studies. Contractility studies were conducted in a manner similar to that described for basilar artery. The vessels were constricted with norepinephrine (1μM) and concentration effect curves for neomycin were obtained for normal and vasospastic arteries.

The surgical procedures were done by Dr. J. F. Megyesi from the Division of Neurosurgery.

2. PREPARATION OF OXYHB

OxyHb was prepared from commercially available human hemoglobin (Sigma) by a modification of the method described by Martin et al., (1985). Since MetHb heavily contaminates the commercially available hemoglobin, this method aims at reducing MetHb to OxyHb using dithionate as a reducing agent. Briefly, sodium dithionate (10mM) was added to the 1mM solution of hemoglobin then this

reducing agent was removed by extensive dialysis, using a 1000 volume of 0.9% NaCl, at a temperature of 2-8°C, for six hours. The purity of OxyHb was then determined by absorption spectrophotometry. The aliquots were stored at a temperature of -80°C.

II. CELL CULTURE

Cynomolgous monkey cerebrovascular smooth muscle cells prepared previously in our laboratory using a modification of the method by Ross (1971) were used in these studies. Briefly, middle cerebral arteries were isolated from cynomologus monkeys under sterile conditions. The arteries were placed in a dish containing Dulbecco's modified Eagles Medium (DMEM). The arteries were then cut in 5mm segments and the adventitia peeled off and then the vessel segments were cut open along their longitudinal axis. The endothelium was removed by gentle scrapping. The artery segments were then chopped into 1-2mm cubes and transferred to 25-sq culture flasks containing DMEM supplemented with 10% fetal calf serum, penicillin (100U/ml) and streptomycin (100μg/ml). Explants were kept in tissue culture incubator at 37°C in humidified air containing 5% CO_2. After the explants became attached to the flasks, the volume of the medium was made up gradually to 4 ml per flask over a period of 4 days. The culture medium was changed weekly under sterile conditions and when the cells became confluent they were transferred to 75-sq cm flasks . Cells were subsequently subcultured at a ratio of 1 : 3 . Smooth muscle cells showed the characteristic " hills and valleys " pattern of growth (Vollrath et al., 1994) .Passages 10 to 16 were used in this study.

III. DETERMINATION OF INTRACELLULAR FREE CALCIUM

Smooth muscle cells were gently scraped from culture flasks, and then incubated with 5μM/L Fura 2 acetoxymethyl ester (Fura 2 AM) for 45 minutes in Krebs-Henseleit solution. The loaded cells were then washed twice, and resuspended in fresh buffer I

at a density of 10^{6}/ml. Cell suspensions were kept in the dark at 20°C until use. Fluorescence of the samples was measured at 37°C using a fluorescence spectrophotometer (Perkin-Elmer model MKF). Fluorescence was measure at emission wave lengths 505nm and excitation wave length at 340 and 380nm. The spectrophotometer was equipped with a stirring apparatus for cell mixing. Values for intracellular free calcium were calculated according to the method of Grynkiewicz et al., (1985) using the equation

$$[Ca^{2+}] = K_d\left(\frac{R - R_{min}}{R_{max} - R}\right)\left(\frac{S_{f2}}{S_{b2}}\right)$$

R_{min} and R_{max} were determined by treatment with x100 Triton 10% in the presence of 5mM EGTA, and 5mM calcium chloride, respectively.

1. EXPERIMENTAL PROTOCOL

These studies were carried out in cells exposed to OxyHb (1μM) for 48 and 72 hours . The aminoglycosides (5mM) were added to the cells 3 hours after the addition of OxyHb, since this would be expected to mimic vasospasm . The culture medium was changed every 24 hours and fresh solutions of OxyHb and aminoglycosides were added.

In the second set of experiments the cells were exposed to OxyHb (1μM) for 24 hours and the aminoglycosides were added 3 hours after the addition of

OxyHb, at the concentrations equal to the $IC_{50's}$ determined from contractility studies with OxyHb.

IV. STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of the mean. Statistical significance was assessed using Student's paired t test for experiments with the carotid arteries when only two groups were compared. In experiments comparing more than two groups, statistical significance was assessed using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test when significant probability was reached. A probability of less than 0.05 ($P<0.05$) was considered significant Statistics were done using Instat 2.1® computer program and graphs were done using Microsoft Excel. 5. computer program.

CHAPTER THREE

RESULTS

I. CONTRACTILITY STUDIES

1. VASORELAXANT EFFECTS OF AMINOGLYCOSIDES ON CONSTRICTION OF BASILAR ARTERY INDUCED BY OXYHB

OxyHb is a major spasmogen implicated in vasospasm, which produces vasoconstriction by mechanisms that are not yet clear but may involve activation of phospholipases, elevation of intracellular calcium level and activation of PKC. Therefore, if activation of PLC is involved, inhibitors of this enzyme would be expected to attenuate the effects of OxyHb. We have thus used neomycin, a well known inhibitor of PLC to examine the action of this compound and related aminoglycosides with similar structural characteristics, on canine basilar artery. OxyHb administered at a single concentration (10μM) produced a slowly developing and sustained contraction. Neomycin and related aminoglycosides, gentamicin, streptomycin and kanamycin were administered in increasing, cumulative concentrations to preparations in which a tonic contraction to OxyHb had developed. Cumulative concentration effect curves for the tested aminoglycosides are shown in Fig. 4. Cumulative concentrations of neomycin induced complete relaxation 100% (n=6) while the maximal relaxing effects of gentamicin and streptomycin, administered in the same milimolar concentrations were 96.8%±1.86 (n=12) and 86.15%±3 (n=7), respectively. Kanamycin was the least effective vasorelaxant. This aminoglycoside produced 63.4%±5.61 (n=5) relaxation. IC_{50} values for different aminoglycosides against contraction produced by OxyHb are shown in Fig. 5 and in Table 1. The order of potency of aminoglycosides in contractile preparations against OxyHb was neomycin (0.46±0.1mM) > gentamicin (0.53±0.08mM) > streptomycin (1.6±0.29mM) > kanamycin (3.88±0.46mM). This order of potency reflects the number of positive charges; neomycin and gentamicin, which have 6 and 5 ionizable amino groups ,

respectively, were the most potent, while kanamycin and streptomycin which have 4 and 2 amino groups, respectively, were far less effective.

2. VASORELAXANT EFFECTS OF AMINOGLYCOSIDES ON CONSTRICTION INDUCED BY ENDOTHELIN

In the further study the vasorelaxant properties of aminoglycoside antibiotics were tested against contraction induced by endothelin, a potent vasoconstrictor implicated in vasospasm. The expression of endothelin has been reported to be elevated after exposure of smooth muscle cells to OxyHb, an observation which indicate that a part of action of OxyHb may be mediated by the expression of this peptide. Thus, it was important to determine whether aminoglycosides also exert vasorelaxant effects on constrictions induced by endothelin. Cumulative concentration-effect curves for the aminoglycosides examined against constriction produced by endothelin-1 are shown in Fig. 6.
The range of milimolar concentrations used in these experiments was the same as that used in the experiments with OxyHb. The relaxation of the endothelin-induced contraction produced by all tested aminoglycosides was concentrationdependent. Maximal relaxation produced by neomycin and gentamicin was 97%±2 (n=5) and 97.8±1.4 (n=5), respectively. Streptomycin and kanamycin were less effective and produced 88%±4.5 (n=3) and 68%±7 (n=5) maximal relaxation, respectively. IC_{50} values for aminoglycosides against contractions induced by endothelin-1 are shown in Fig. 7 and in Table 1. The order of potency of aminoglycosides against contractions induced by endothelin was similar to that for OxyHb: neomycin and gentamicin were the most potent with $IC_{50's}$ 0.63±0.08 mM, and 0.54±0.05 mM, respectively, streptomycin was less effective 1.88±0.46 mM and kanamycin least effective 2.34±0.92mM.

3. VASORELAXANT EFFECTS OF AMINOGLYCOSIDES ON PGF2α-INDUCED CONSTRICTION

It has been suggested that in addition to OxyHb and endothelin, prostaglandins may play a role in the pathogenesis of vasospasm. PGF2α is a prostaglandin which produces its contractile effects via G-protein coupled receptor and the activation of PLC is a key step in the development of vasoconstriction mediated by this compound. I have thus examined the action of neomycin, which is known to inhibit PLC, and its analogues, on the contractions of basilar arteries produced by PGF2α. Cumulative concentration effect curves for neomycin, gentamicin, streptomycin and kanamycin are shown in Fig. 8. The range of aminoglycoside concentrations used is identical to those for OxyHb and endothelin-1. Maximal relaxations for neomycin, gentamicin, streptomycin and kanamycin were 96.6%±3.12 (n=6), 94.14%±2.5 (n=7), 84.7±6.(3) and 59.4±8.6 (n=4), respectively. The $IC_{50's}$ for these aminoglycosides were : 0.89±0.21mM, 0.67±0.07mM, 0.9±0.09mM and 3.72±0.64mM, respectively (Fig. 9). This order of potency confirms the observations made with OxyHb and endothelin, that the effectiveness of aminoglycoside antibiotics is related to the number of their positive charges.

4. EFFECTS OF AMINOGLYCOSIDES ON CONSTRICTION INDUCED BY RECEPTOR-MEDIATED ACTIVATION OF PLC, IN CALCIUM FREE MEDIUM

Neomycin has been reported to antagonize both PLC activation and calcium influx. To discriminate between these two actions, the experiments were conducted in which the relaxant action of neomycin on the constriction to PGF2α, was examined in calcium free medium. Hydrolysis of

phosphatidylinositol (4,5) bis-phosphate by PLC is known to be independent of increases in intracellular calcium. However, calcium influx is dependent on extracellular calcium. Thus, in these experiments the basilar artery preparations were equilibrated in Ca^{2+} containing medium, and then Ca^{2+}-containing medium was removed and replaced with Ca^{2+} free Krebs-Henseleit buffer, containing 2 mM EGTA, an extracellular calcium chelating agent. The absence of calcium in the medium was confirmed using KCl, known to produce vasoconstriction by stimulating Ca influx. In these experiments KCl did not produce any contraction, thus confirming that the buffer was Ca^{2+} free. The representative traces of the effect of PGF2α and the relaxant effects of neomycin are shown in Fig. 10. The ability of neomycin to relax vasoconstriction in calcium free medium suggests that PLC inhibition is the mechanism of action of neomycin, independent on its action on calcium influx.

5. VASORELAXANT EFFECTS OF AMINOGLYCOSIDES ON KCL-INDUCED CONSTRICTION

Since neomycin has been reported to inhibit calcium channels in various preparations it was of interest to see whether this agent can produce this effect in cerebral arteries. In these experiments high depolarizing concentrations (60mM) of KCl, known to activate voltage dependent calcium channels (L-type), were used. Cumulative concentration-effect curves for the aminoglycosides examined, are shown in Fig. 11. All tested aminoglycosides (neomycin, gentamicin, streptomycin and kanamycin) relaxed constriction to KCl. The maximal relaxant effect for neomycin and gentamicin were 95.6%±1.28 (n=5) and 96.8%±3.2 (n=4), respectively. The maximal effects mediated by streptomycin and kanamycin, observed at the same molar concentrations, were 69.8% ±6.9 (n=5) and 52.8%((n=5), respectively. The IC_{50s} for aminoglycosides

were 0.86±1.4, 1.12±0.14, 2.34±0.75 and 3.74±0.55 for neomycin, gentamicin, streptomycin and kanamycin respectively (Fig. 12). This indicates that the most potent agents were neomycin and gentamicin, while streptomycin and kanamycin required much higher molar concentrations for their relaxant actions. These experiments also show that in addition to their inhibitory action on PLC these agents also inhibit calcium influx mediated via voltage dependent calcium channels.

6. THE EFFECTS OF AMINOGLYCOSIDES ON CONSTRICTION MEDIATED BY THE ACTIVATION OF PKC

PKC is believed to play a pivotal role in sustained vasoconstriction and hence in cerebral vasospasm. As mentioned earlier, the signaling pathways activated by OxyHb, endothelin and PGF2(involve the DAG generation and PKC stimulation. In these experiments, the ability of phorbol esters to activate PKC directly, was utilized to investigate the actions of aminoglycosides on the contraction mediated by the activation of PKC. Fig. 13 shows that neomycin, gentamicin, streptomycin and kanamycin, added in the same molar concentrations which were used in previous experiments, all produced relaxation of PMA mediated constriction. The maximal relaxations induced by neomycin, gentamicin and streptomycin were 88%±5.79 (n=6), 81%±5.4 (n=5) and 87%±4.7 (n=5), respectively, while kanamycin was much less effective 52%±8.7 (n=4). The $IC_{50's}$ were 1.63-±0.27, 1.54±0.34, 1.43±0.35 and 4.51±0.48 for neomycin, gentamicin, streptomycin and kanamycin, respectively (Fig. 14). These experiments suggest that, at least a part, of the vasodilatory action of aminoglycosides on vasoconstriction, is mediated by the inhibition of PKC.

7. TIME-COURSE OF THE VASORELAXANT EFFECT OF AMINOGLYCOSIDES

Since vasospasm is a condition in which cerebral arteries are constricted for days and sometimes weeks it was of interest to examine whether the effects of aminoglycosides are transient or maintained for long time. Fig. 15 shows experimental traces of the effects of kanamycin on canine basilar artery followed over a time period of three hours. Similar results were obtained using other antibiotics (results not shown). In these experiments, control vessels exposed to OxyHb showed a sustained constriction that was maintained for at least three hours of the experimental protocol. Cerebral arteries in which aminoglycosides, neomycin, gentamicin, streptomycin and kanamycin, were administered on top of tension induced with OxyHb, showed relaxation that was maintained for three hours of observation. In the end of the experiment, this vasorelaxant effect was characterized by the same or very similar magnitude as the initial vasodilatory effects. These experiments have provided evidence that the vasorelaxant effects of aminoglycosides on OxyHb-induced vasoconstriction, are sustained for at least three hours.

8. EFFECTS OF ENDOTHELIUM REMOVAL ON NEOMYCIN-INDUCED VASORELAXATION

The endothelium is a source of nitric oxide, known to exert a relaxant effect on blood vessels. The involvement of nitric oxide in vasorelaxation is usually studied in the presence and in the absence of endothelium, to show that the presence of endothelium is required for nitric oxide-mediated action on blood vessels. This approach was used in these studies to examine whether or not the vasorelaxant effects of neomycin are endothelium dependent. The absence of endothelium was confirmed pharmacologically using bradykinin, an agonist

known to produce an endothelium dependent relaxation. Fig. 16A shows experimental recording traces obtained using canine basilar artery rings preconstricted with OxyHb (10μM) or PGF2α (1μM) (Fig. 16B), in which cumulative concentrations of bradykinin were added. As shown in Fig. 16A and 14B no vasorelaxant effects were observed after administration of bradykinin, on the contrary, only contractile effects were observed with this agent, thus confirming the absence of endothelium. In the same preparations neomycin (5mM) produced relaxation indicating that its vasorelaxant effect is endothelium independent.

9. COMBINED ACTIONS OF SUBMAXIMAL CONCENTRATIONS OF NEOMYCIN AND PAPAVERINE

Papaverine, which is a nonselective inhibitor of cyclic nucleotide-dependent intracellular phosphodiesterases and hence is a smooth muscle relaxant, has a role in the treatment of cerebral vasospasm. However, the usefulness of this agent is limited by its transient effects. Therefore, it was of interest to determine whether papaverine and neomycin, as a representative of this group, may have additive effects. The combined effects of the subeffective concentrations of both agents are shown in Fig. 17.

The percentage of vasorelaxation produced by neomycin (1mM) and papaverine hydrochloride (1μM) administered separately was 33.6±7.1 (n=5) and 24.4%±4.9 (n=10), respectively. However, the vasorelaxant effect of the combination of both agents was greater [46.3%±4.77 (n=10)] than the effects of papaverine and neomycin given alone. The difference between the combination and papaverine alone was significant in case of papaverine ($p<0.05$) but did not reach statistical significance for neomycin.

10. THE EFFECTS OF NEOMYCIN ON ARTERIES IN SPASM

It is well documented that the preparations of blood vessels obtained from patients after SAH and from animal models of vasospasm are characterized by attenuated responsiveness to both contractile and relaxing agents. Therefore, the experiments were carried out to examine whether the relaxant action of neomycin is reduced in vasospastic blood vessels. The preconstricting agent used in this study was norepinephrine, a receptor agonist characterized by powerful and sustained actions on peripheral arteries such as carotid artery. The vasorelaxant effects of neomycin on normal carotid arteries and on spastic arteries, obtained from *in vivo* canine model of vasospasm and preconstricted with norepinephrine, are shown in Fig. 18. The relaxant effects of neomycin were significantly higher in normal vessels at the concentrations of this agent 0.5, 1.0 and 2.0mM ($p<0.05$). Maximal relaxation, however, was not significantly different in both normal and spastic arteries (82%±4.3 and 75.3%±7.1, respectively). Thus, this study has provided evidence that neomycin and probably other aminoglycosides are capable of relaxing of normal as well as vasospastic arteries.

II. STUDIES OF INTRACELLULAR CALCIUM LEVELS

Earlier work from our laboratory has shown that OxyHb induces sustained elevation of intracellular calcium in cultured cerebrovascular smooth muscle cells and that this increase in calcium can be prevented or reversed by neomycin (Vollrath *et al.*, 1994). Therefore, in these studies we asked the following question:

Are the effects of neomycin on intracellular calcium levels stimulated by OxyHb, shared by other aminoglycosides?

Does the effectiveness of aminoglycosides in attenuating intracellular calcium elevation, parallel their effectiveness as vasorelaxants?

Do the inhibitory effects of aminoglycosides on intracellular calcium levels correspond in time to vasospasm?

1. EFFECTS OF AMINOGLYCOSIDES ON INTRACELLULAR CALCIUM LEVELS IN CEREBROVASCULAR SMOOTH MUSCLE CELLS EXPOSED TO OXYHB FOR 24 HOURS

To answer these questions, cultured monkey cerebrovascular smooth muscle cells were exposed to OxyHb for 24 hours and the aminoglycosides, neomycin, gentamicin, streptomycin and kanamycin, were administered three hours later, in concentrations corresponding to the IC_{50s} determined in the contractility studies with OxyHb.

As shown in Fig. 19, OxyHb stimulated an increase in the level of intracellular calcium [450±31.8 nM (n=8)], which was significantly different from control ($p<0.001$). Neomycin and its analogues, gentamicin, streptomycin and kanamycin, effectively decreased intracellular calcium levels elevated by OxyHb, to 221±35.6 nM (n=7), 279.2±31.3 nM (n=7), 229.1±33.82 nM (n=6) and 240±59.8 nM(n=5), respectively. These decreases were significantly different from OxyHb-induced calcium rise, with the $p<0.01$, <0.05, <0.01 and <0.05 for neomycin, gentamicin, streptomycin and kanamycin, respectively. There was no significant difference between the effects of any of the aminoglycosides and the control, nor was there significant difference among the effects of individual aminoglycosides.

When calcium levels, obtained after treatment with aminoglycosides, were expressed as a percentage of the calcium level elevated by OxyHb, it became apparent that the $IC_{50's}$ of all aminoglycosides produced approximately 50% inhibition of the intracellular calcium elevation induced by OxyHb (49.1±8%, 62±7%, 50±7.5% and 53±13.3% for neomycin, gentamicin, streptomycin and

kanamycin, respectively). Thus, these results strongly suggest that the ability of the aminoglycosides to inhibit intracellular calcium accumulation parallels their vasorelaxant effects.

2. TIME-COURSE OF CHANGES IN THE LEVELS OF INTRACELLULAR CALCIUM MEDIATED BY OXYHB AND AMINOGLYCOSIDES

Exposure of cerebrovascular smooth muscle cells in culture to OxyHb can be used as an *in vitro* model of vasospasm for studying the long term effects of potential therapeutic agents. We have thus examined the ability of the aminoglycosides to inhibit the sustained elevation of calcium level which occurs after exposure to OxyHb over long time period.

Results in Fig. 20 show that the intracellular calcium levels in cells exposed to OxyHb for 24, 48 and 72 hours were 534.1(53 nM (n=25), 670±73.5 nM (n=11) and 959 (156 nM (n=8), respectively. The increases in calcium were significantly different from control ($p<0.01$, $p<0.01$, $p<0.001$ for 24, 48 and 72 hours, respectively). These results confirm that OxyHb causes a steady increase in intracellular calcium over a prolonged time period.

Further study examined the activity of the four aminoglycosides against the changes in intracellular calcium which occur after exposure to OxyHb (1 μM) over 48 hours. Neomycin, gentamicin, streptomycin and kanamycin, each at a concentration of 5mM were administered to cultured cerebrovascular smooth muscle cells three hours after the cells were exposed to OxyHb. The culture medium was changed every day and replaced with fresh medium containing the same concentrations of OxyHb and aminoglycosides. The effects of the aminoglycosides are shown in Fig. 21. Administration of these compounds attenuated elevated calcium to levels which are not significantly different from untreated control [210±38% (n=5), 181±28% (n=4), 216.±49% (n=5) and

186±24% (n=7) for neomycin, gentamicin, streptomycin and kanamycin, respectively].

In an attempt to mimic cerebral vasospasm similar experiments were conducted with the time course extended to 72 hours. In these experiments, the aminoglycosides (5 mM) were administered to cerebrovascular smooth muscle cells three hours after exposure to OxyHb (1μM) and the culture medium was changed daily as in the experiments done for 48 hours. The effects of aminoglycosides are shown in Fig. 22. As mentioned above, the rise in intracellular calcium stimulated with OxyHb continued for several days. Administration of the aminoglycosides reduced this increase in calcium to control level which was significantly different from that corresponding to OxyHb alone 124%±30% $p<0.001$ (n=5), for neomycin, 140%±.31% $p<0.01$(n=4) for gentamicin, 237%±77% $p<0.01$(n=3) for streptomycin and 137%±28% $p<0.05$ (n=6) for kanamycin. Thus these results suggest that the aminoglycosides are effective inhibitors of intracellular calcium elevation, one of the key processes believed to be involved in the pathogenesis of cerebral vasospasm.

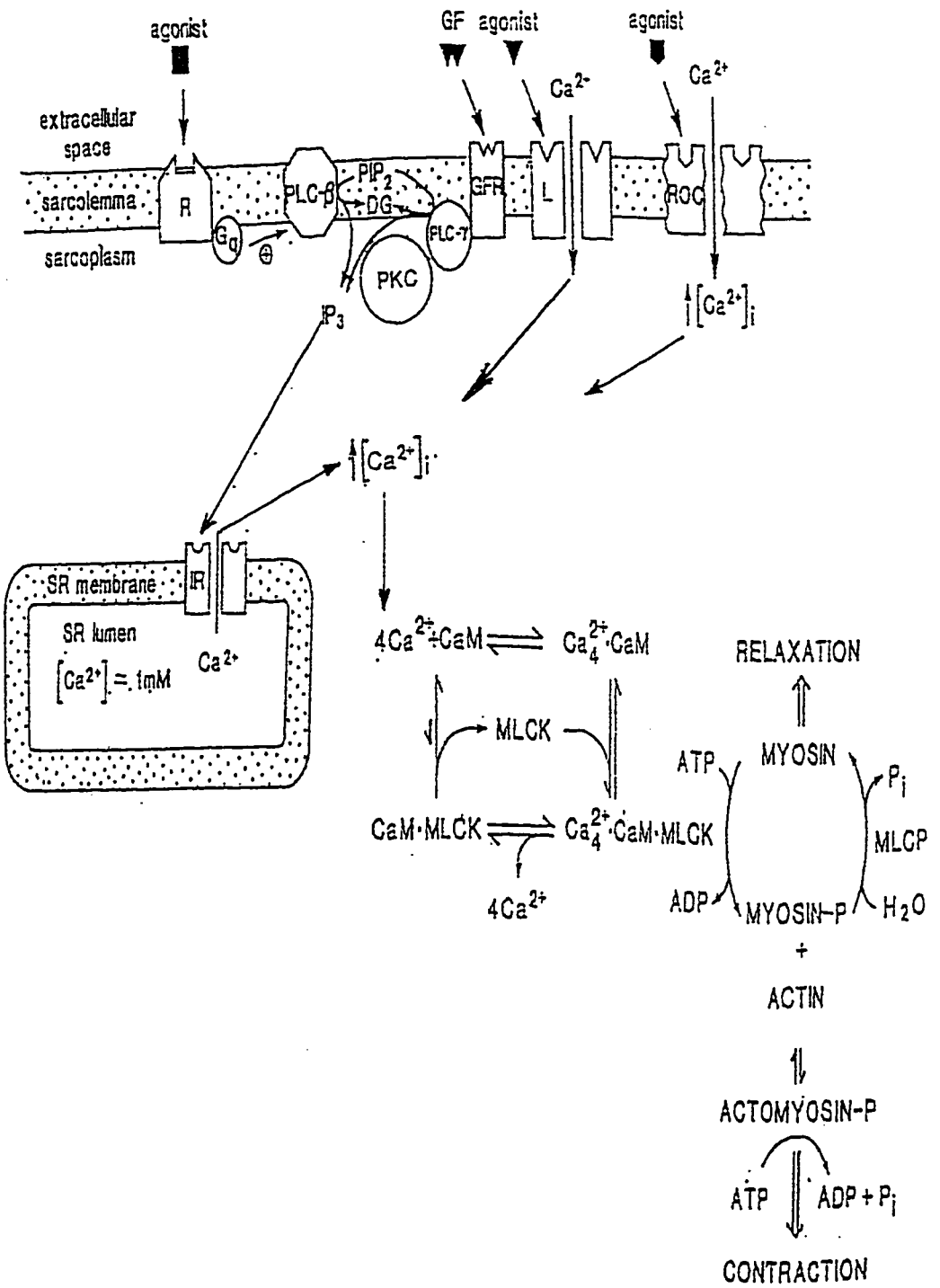
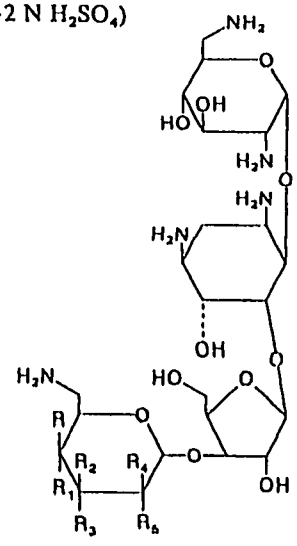
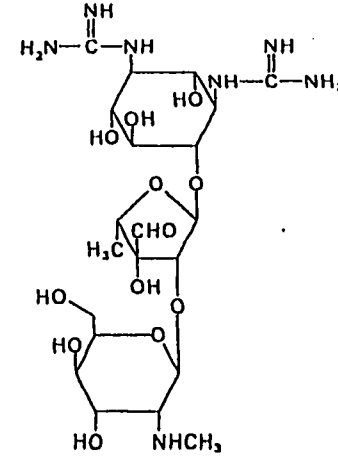


Fig. 1. Signal transduction in smooth muscle cells.

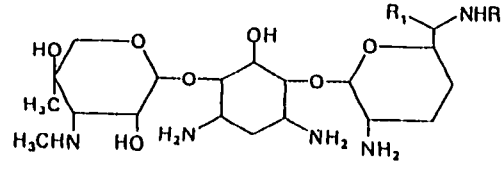
NEOMYCIN B (*Actilin, Soframycin*)
$C_{23}H_{46}O_{13}N_6$
$R = R_3 = OH$; $R_1 = R_2 = R_5 = H$; $R_4 = NH_2$
M.p. Indefinite
$[\alpha]_D^{25}$ +83° (c 1·0, 0·2 N H_2SO_4)



STREPTOMYCIN A
$C_{21}H_{39}O_{12}N_7$
M.p. Indefinite



GENTAMICIN C_1
$C_{21}H_{43}O_7N_5$
$R = R_1 = CH_3$
M.p. 94-100°C
$[\alpha]_D^{25}$ +158° (H_2O)



KANAMYCIN A
$C_{18}H_{36}O_{11}N_4$
$R = NH_2$; $R_1 = OH$
M.p. Not given
$[\alpha]_D^{24}$ +146° (c 1·0, 0·1 N H_2SO_4)

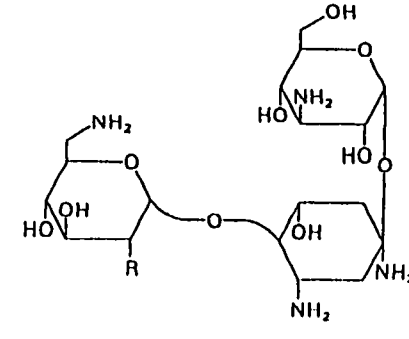


Fig. 2. Chemical structure of the aminoglycoside antibiotics.

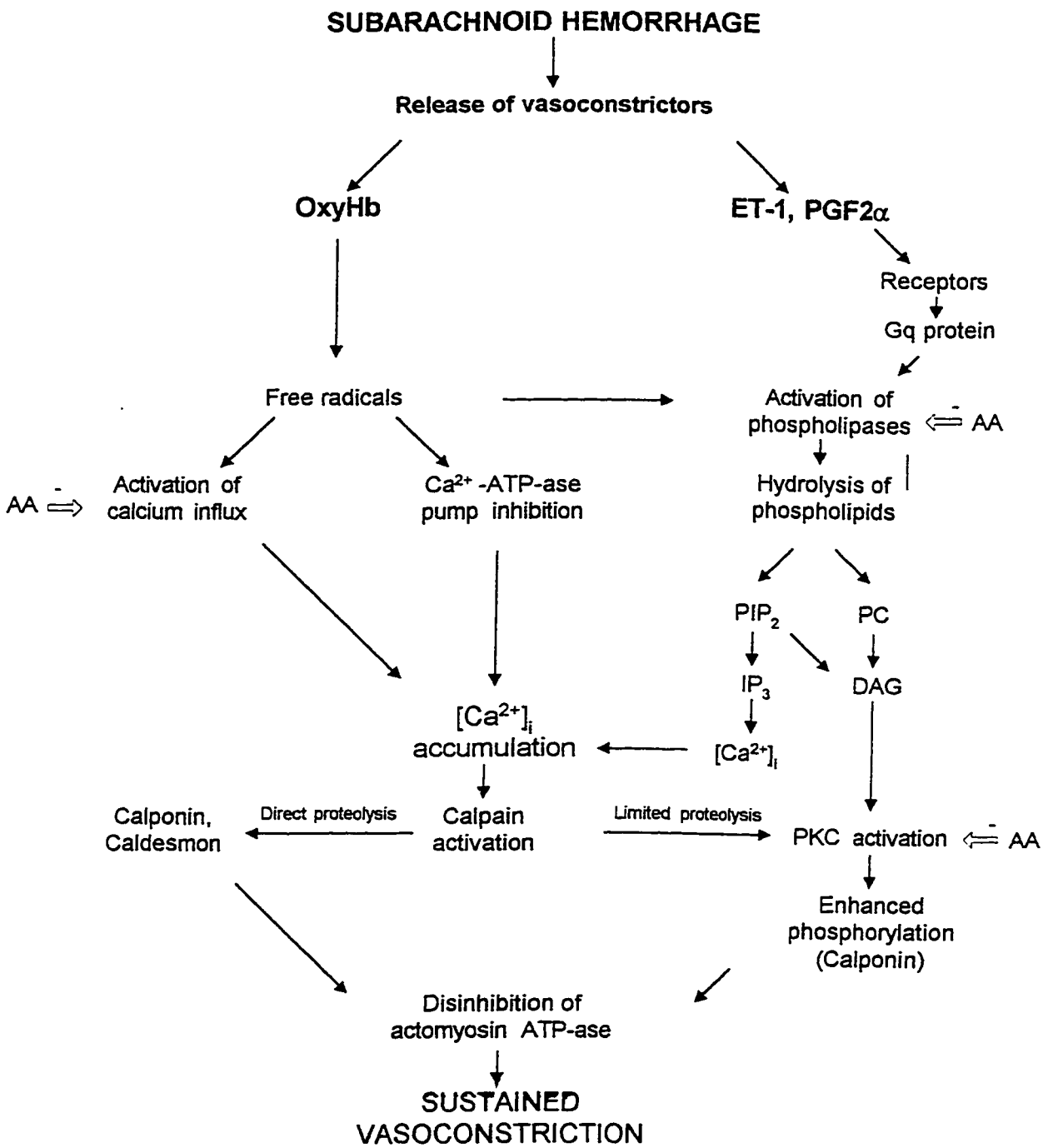


Fig. 3. Possible mechanisms involved in the production of cerebrovascular spasm following subarachnoid hemorrhage

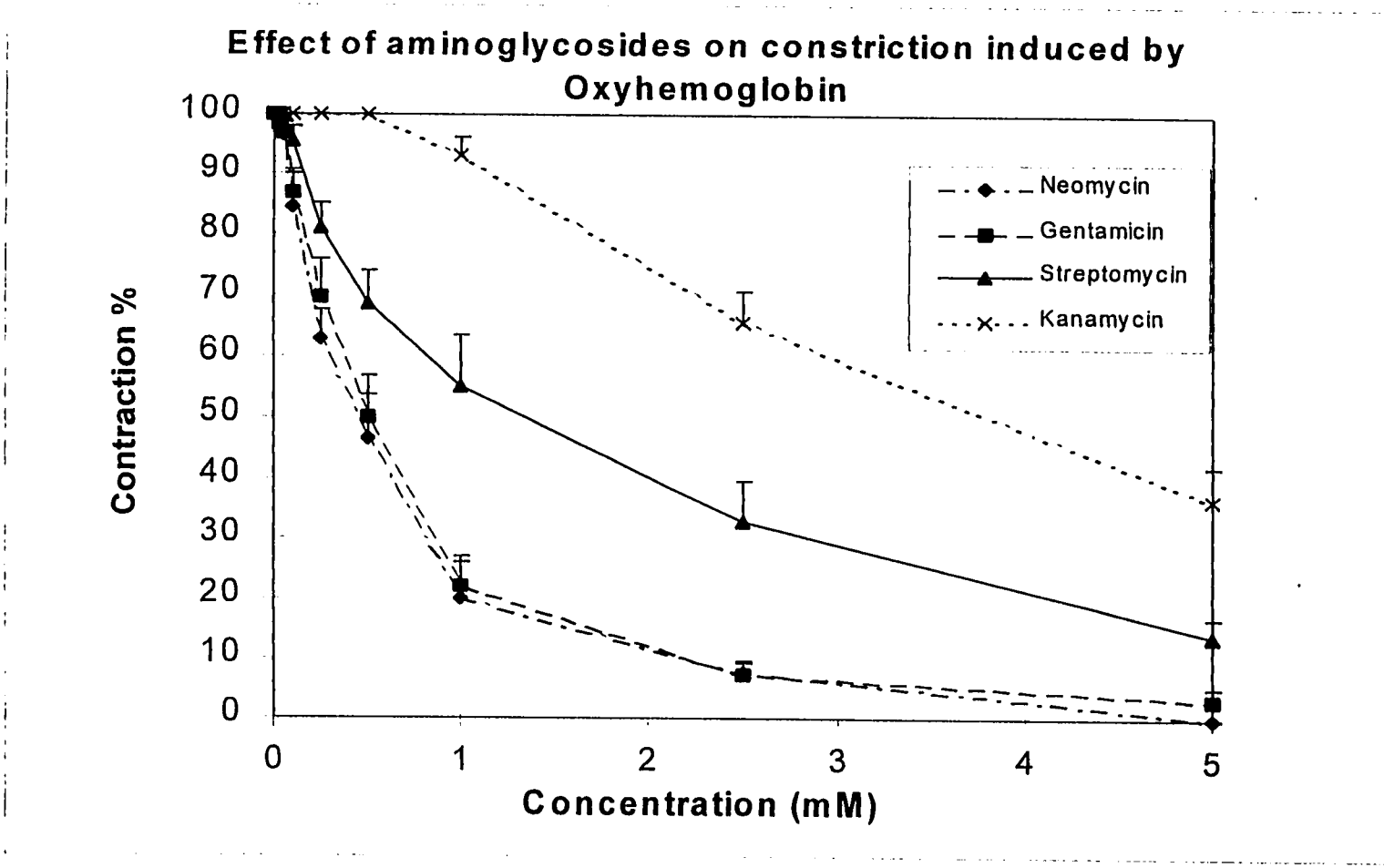


Fig. 4. The vasorelaxant effect of aminoglycoside antibiotics on canine basilar artery rings preconstricted with OxyHb (10μM). Vertical bars indicate SEM (n=5-12).

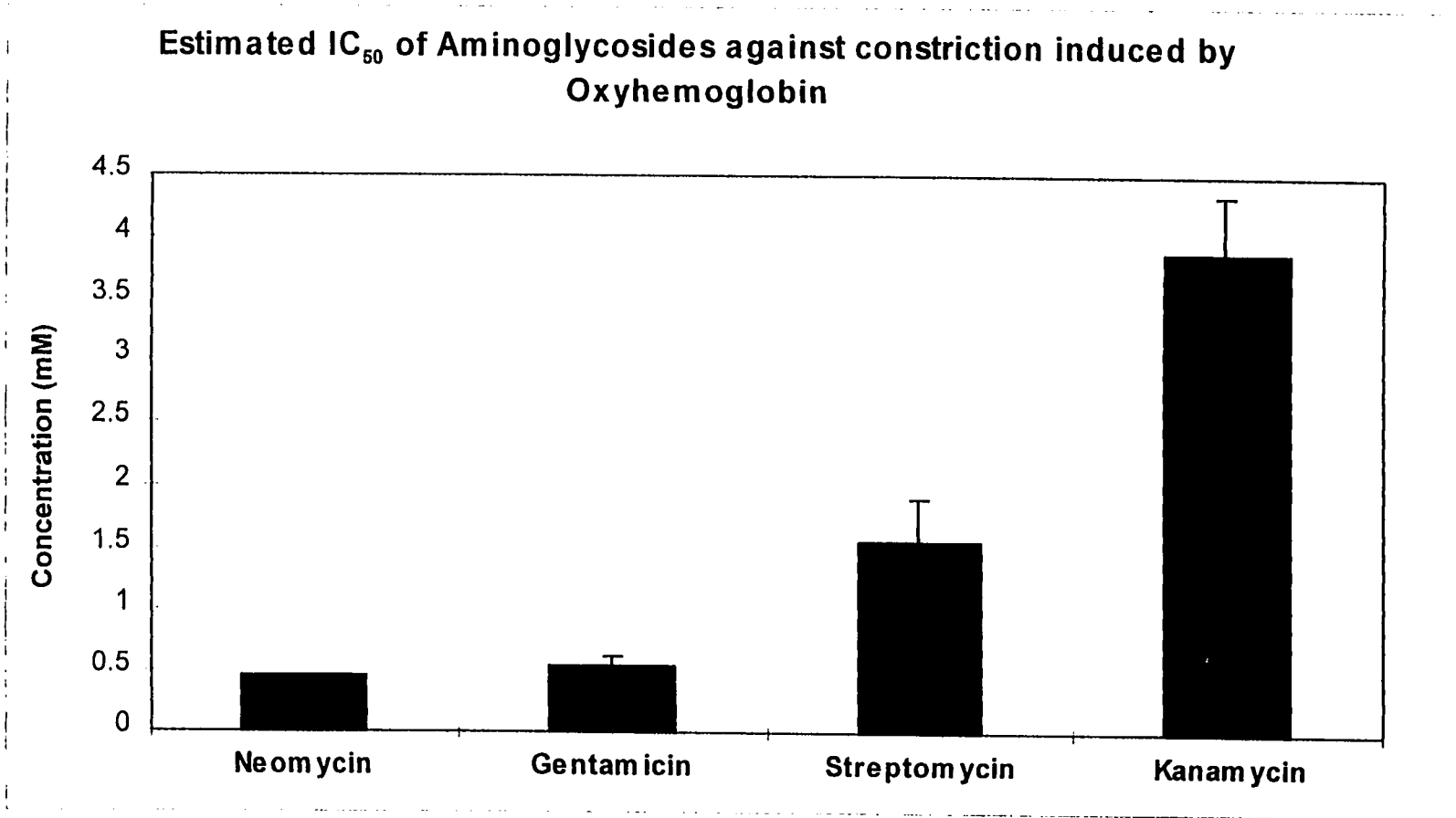


Fig. 5. Estimated $IC_{50's}$ for aminoglycoside antibiotics obtained using canine basilar artery rings preconstricted with OxyHb (10μM). The tonic phase of constriction was considered 100% contraction and relaxation to papaverine (0.5mM) was used as 100% relaxation. Vertical bars indicate SEM (n=5-12).

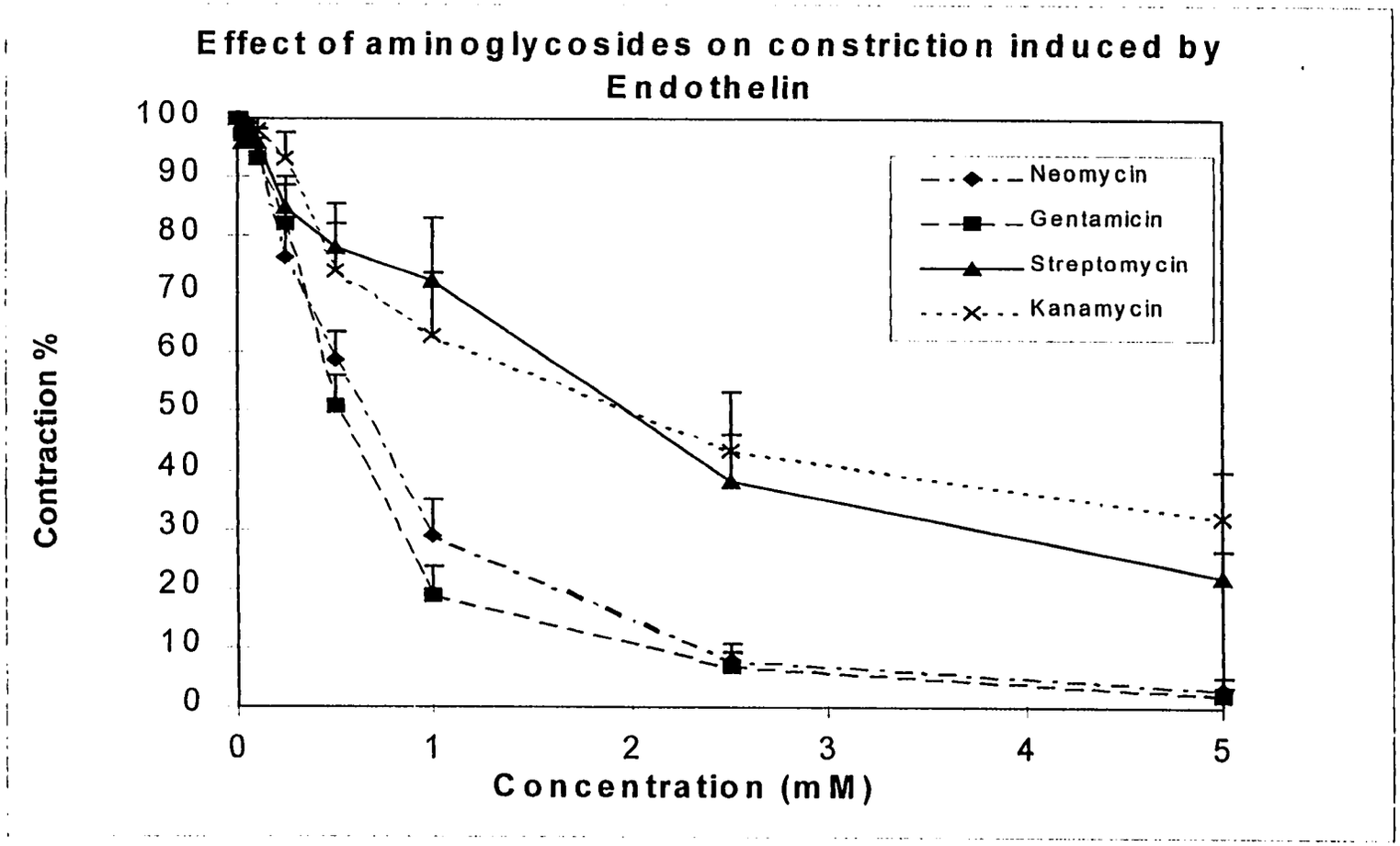


Fig. 6. The vasorelaxant effect of aminoglycoside antibiotics on canine basilar artery rings preconstricted with ET-1 (0.5nM). Vertical bars indicate SEM (n=3-5).

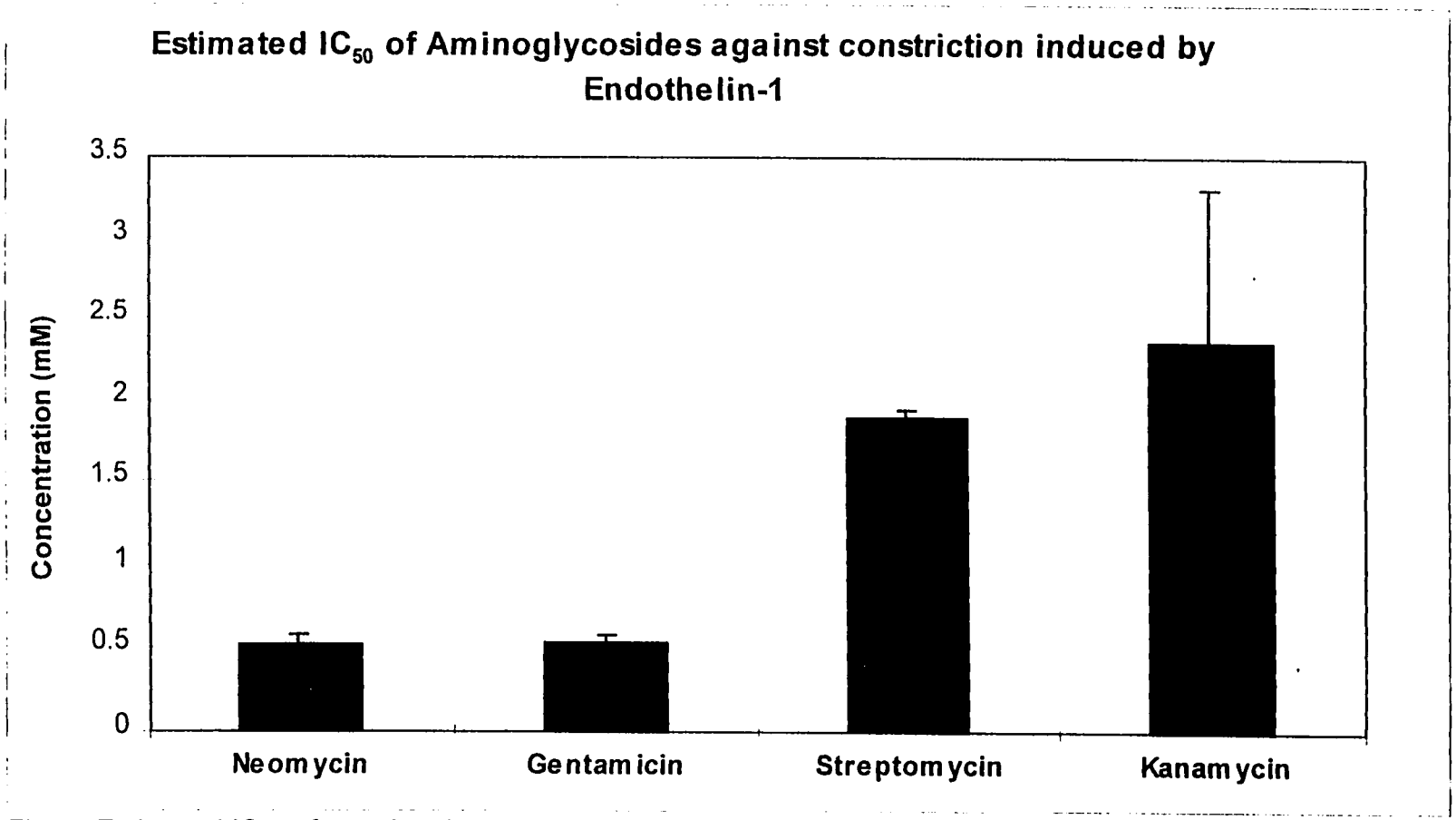


Fig. 7. Estimated $IC_{50's}$ for aminoglycoside antibiotics obtained using canine basilar artery fings preconstricted with ET-1 (0.5nM). The tonic phase of constriction was considered 100% contraction and the relaxation to papaverine (0.5mM) was used as 100% relaxation. Vertical bars indicate SEM (n=3-5).

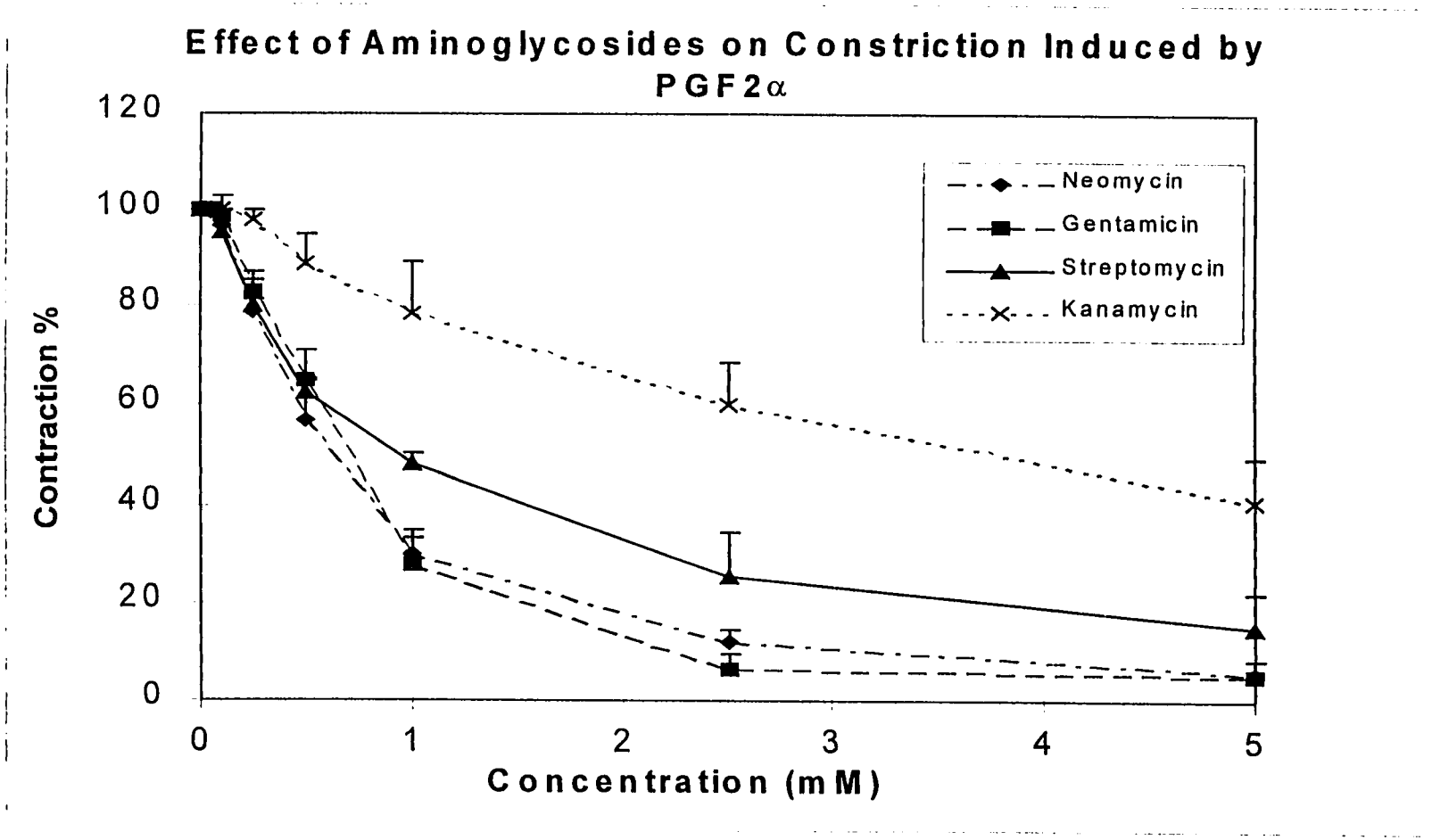


Fig. 8. The vasorelaxant effect of aminoglycoside antibiotics on canine basilar artery rings preconstricted with PGF2α (1μM). Vertical bars indicate SEM (n=3-7).

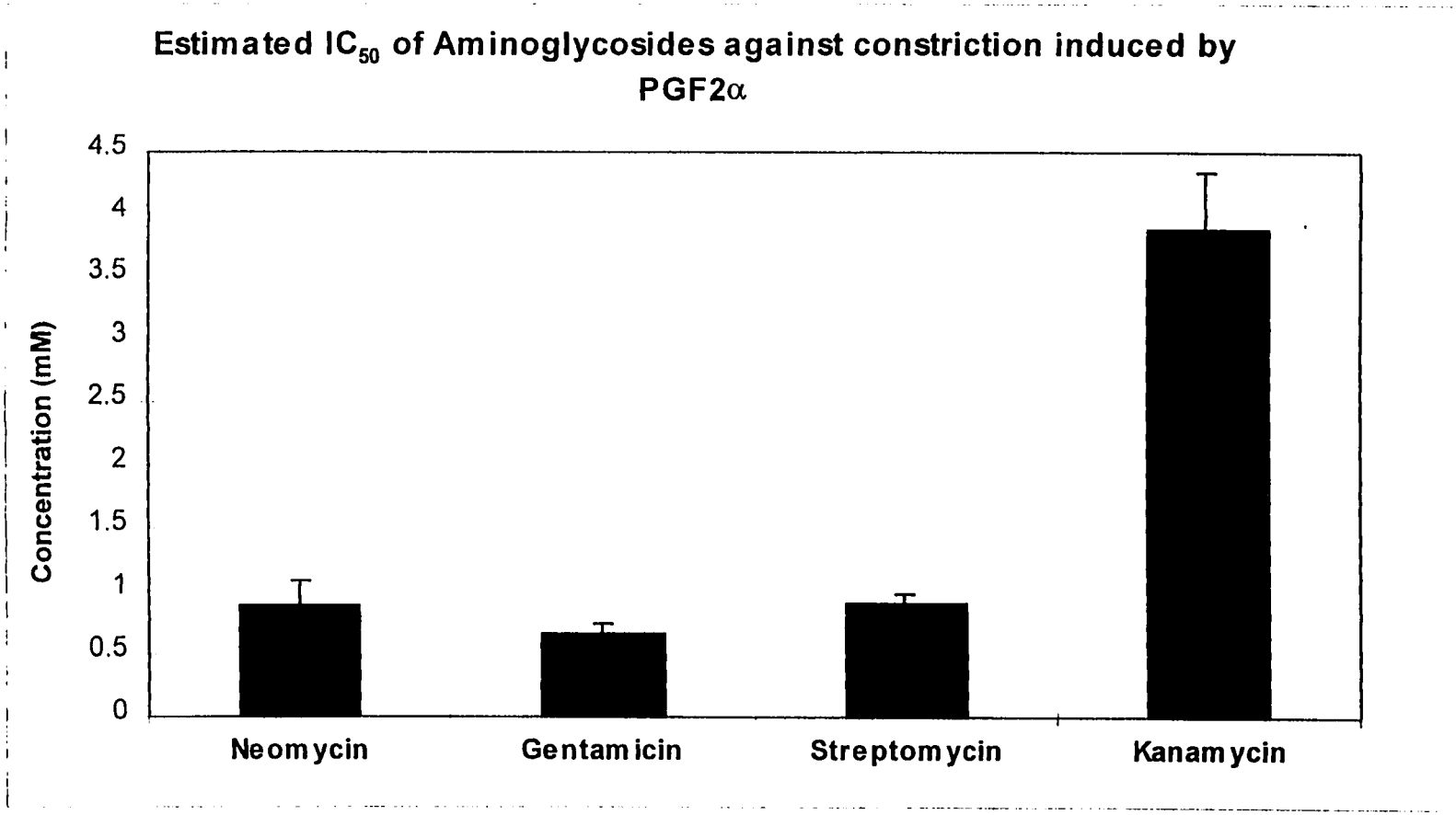


Fig. 9. Estimated $IC_{50's}$ for aminoglycoside antibiotics obtained using canine basilar artery rings preconstricted with PGF2α (1μM). The tonic phase of constriction was considered 100% contraction and relaxation to papaverine (0.5mM) was used as 100% relaxation. Vertical bars indicate SEM (n=3-7).

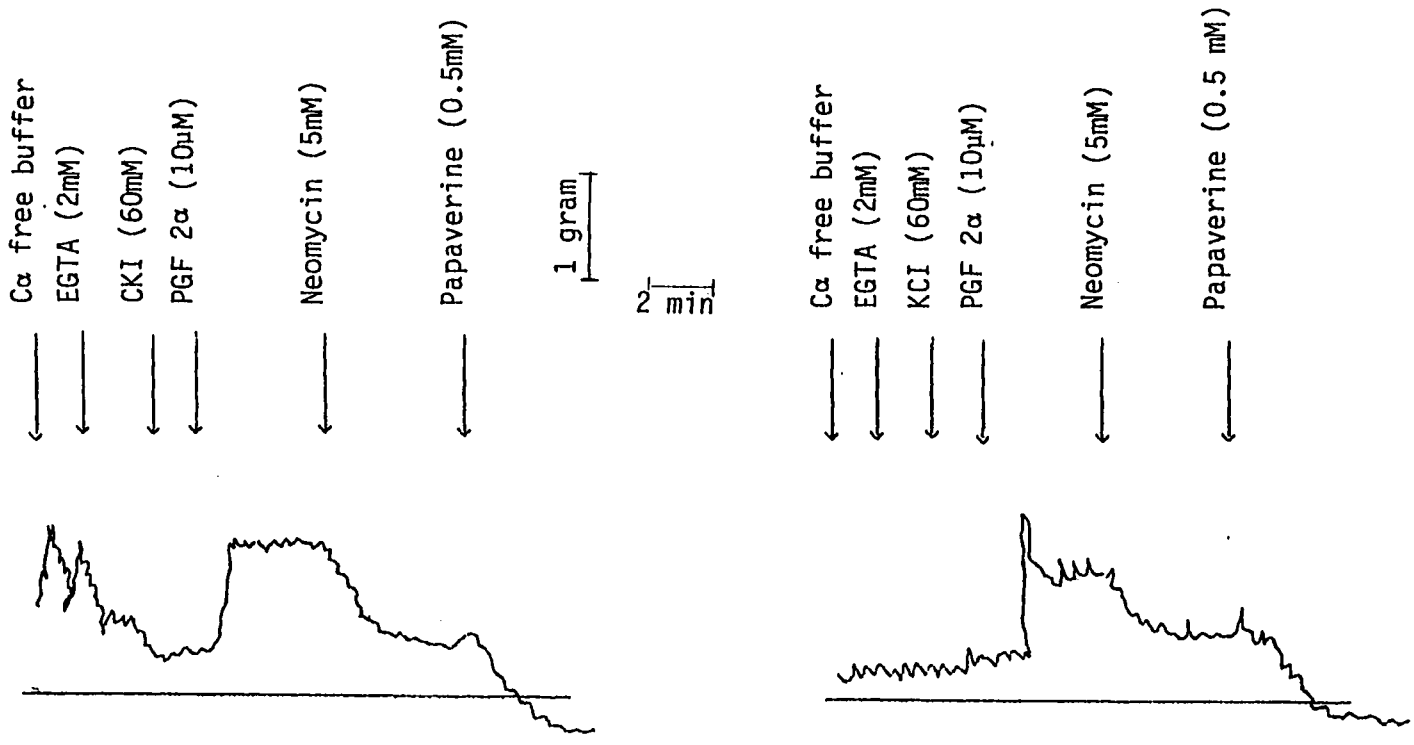


Fig. 10. Representative traces of the effect of neomycin (5mM) on canine basilar artery rings preconstricted with PGF2α (1μM) in calcium free medium.

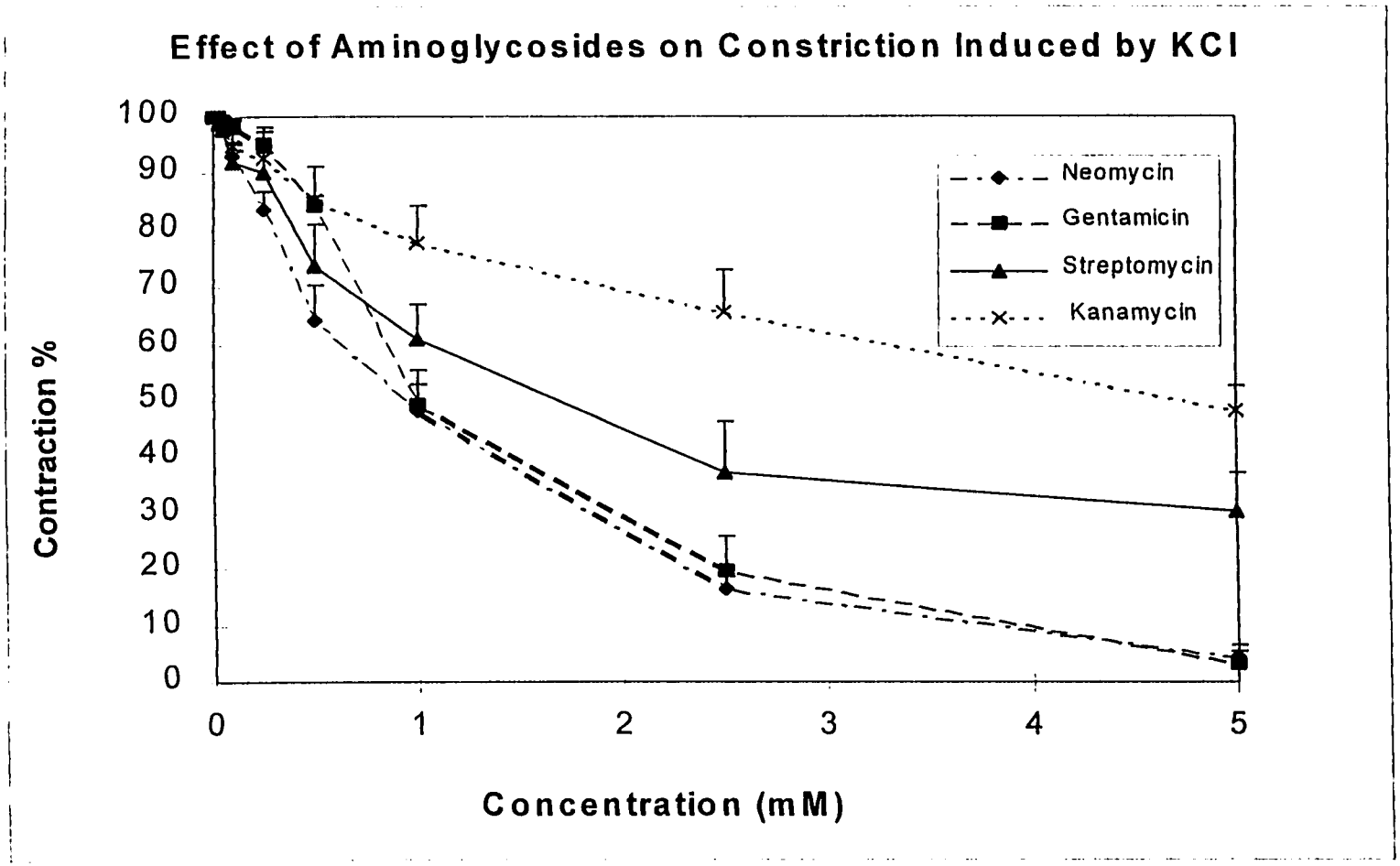


Fig. 11. The vasorelxant effect of aminoglycoside antibiotics on canine basilar artery rings perconstricted with KCl (60mM). Vertical bars indicate SEM (n=4-5).

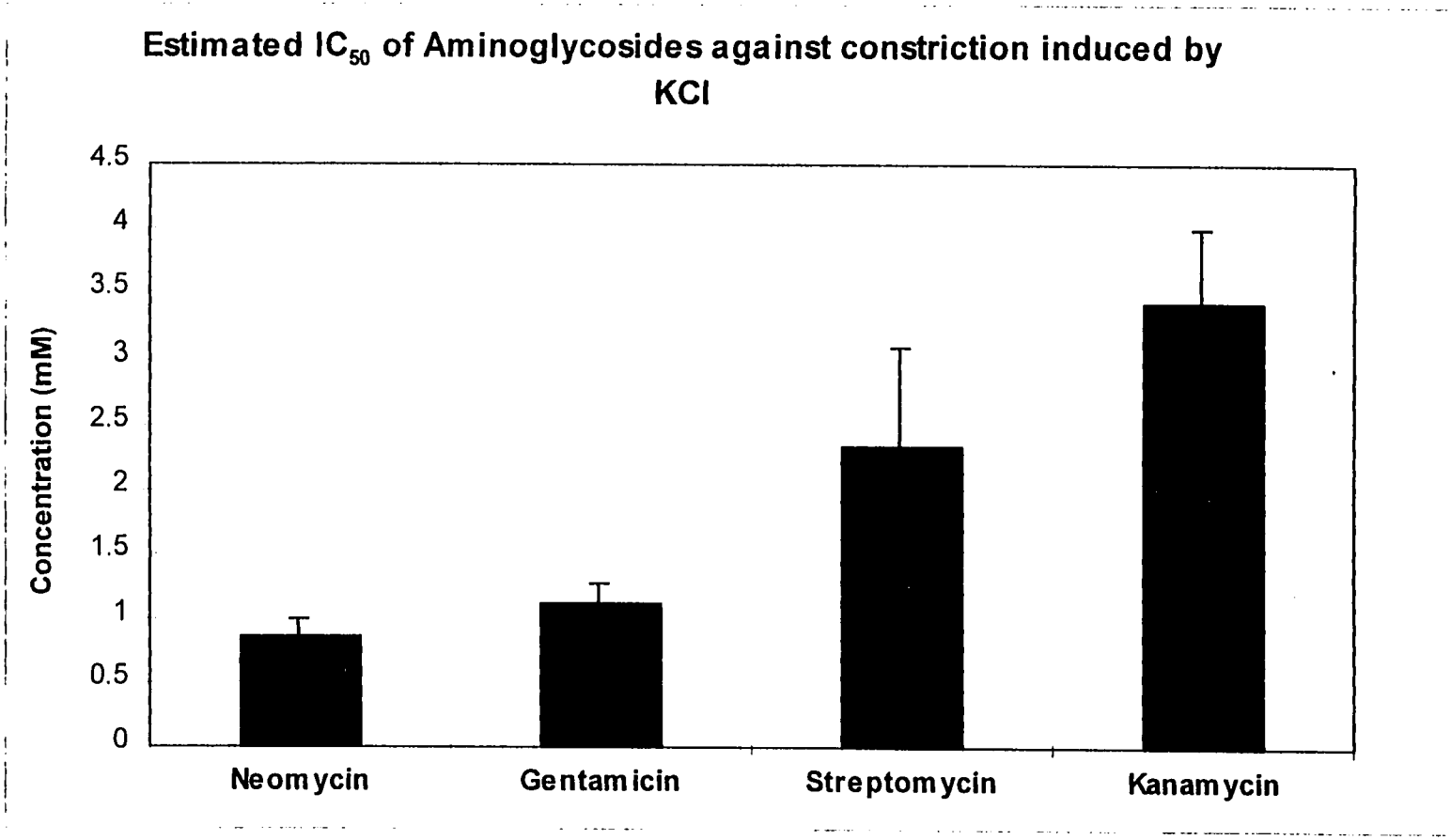


Fig. 12. Estimated $IC_{50's}$ for aminoglycoside antibiotics obtained using canine basilar artery rings preconstricted with KCl (60mM). The tonic phase of constriction was considered 100% contraction and the relaxation to papaverine (0.5mM) was used as 100% relaxation. Vertical bars indicate SEM (n=4-5).

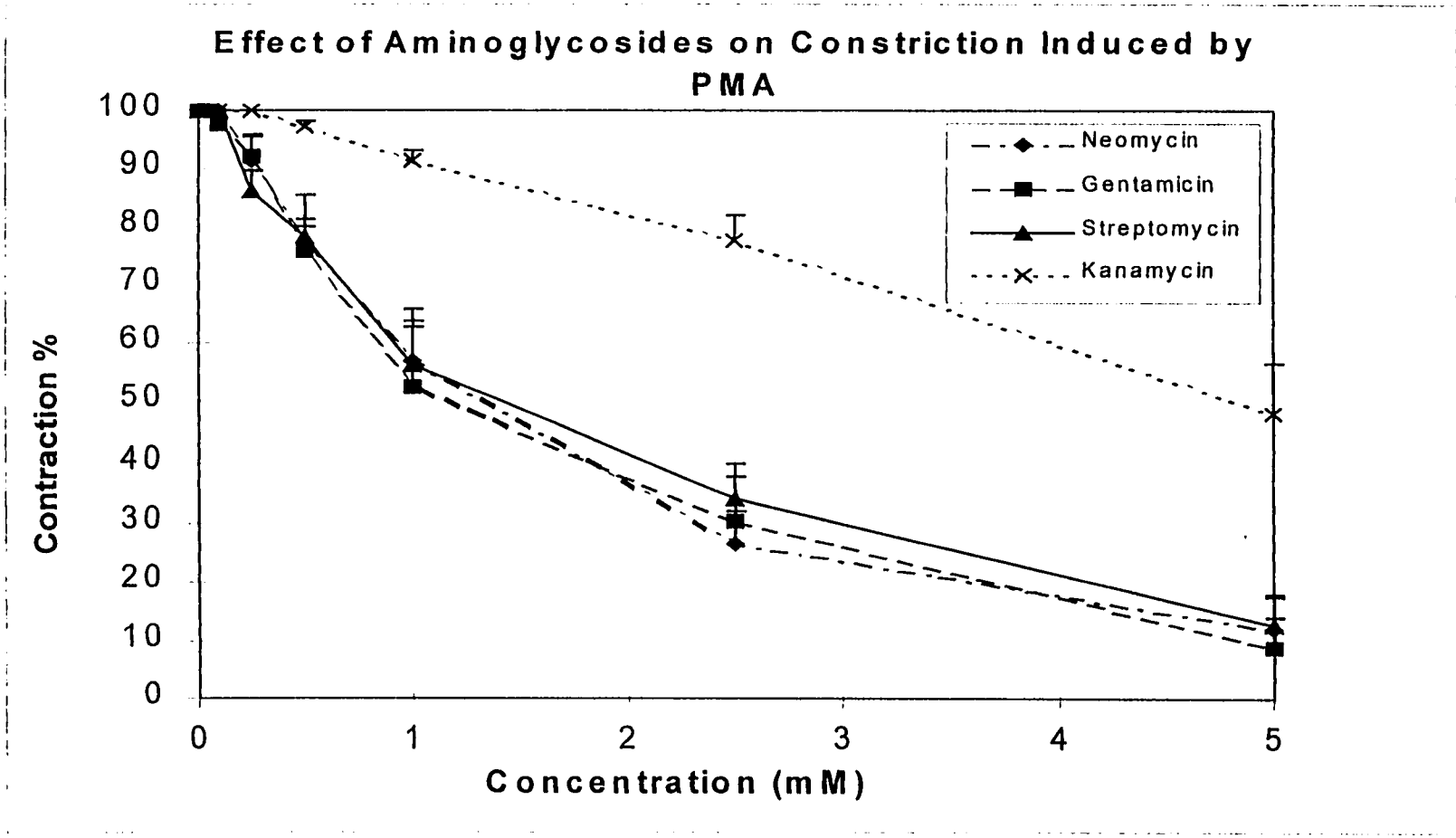


Fig. 13. The vasorelaxant effect of aminoglycoside antibiotics on canine basilar artery rings preconstricted with PMA (160nM).Vertical bars indicate SEM (n=4-5).

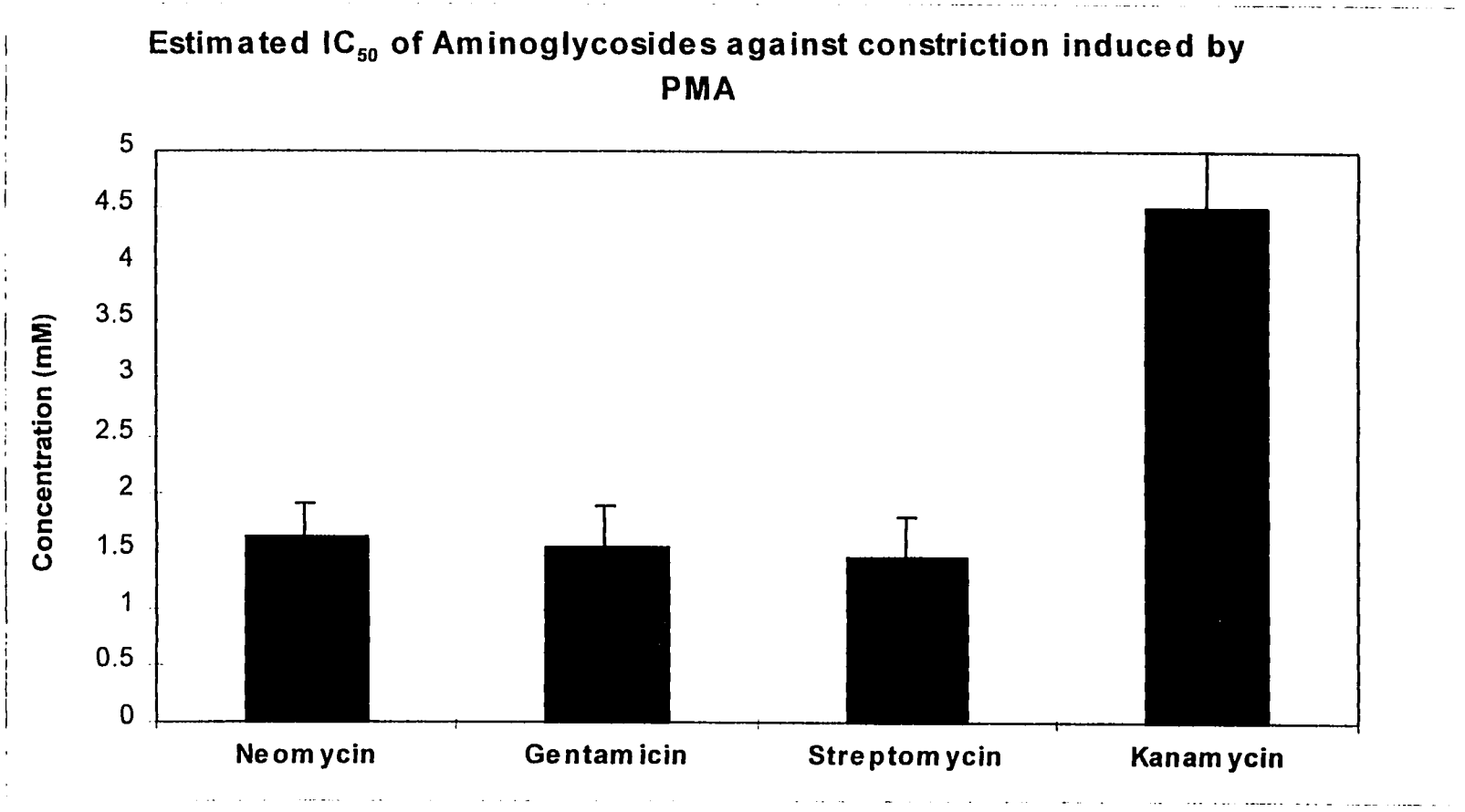


Fig. 14. Estimated $IC_{50's}$ for aminoglycoside antibiotics obtained using canine basilar artery rings preconstricted with PMA (160nM). The tonic phase of constriction was considered 100% contraction and the relaxation to papaverine (0.5mM) was used as 100% relaxation. Vertical bars indicate SEM (n=4-5).

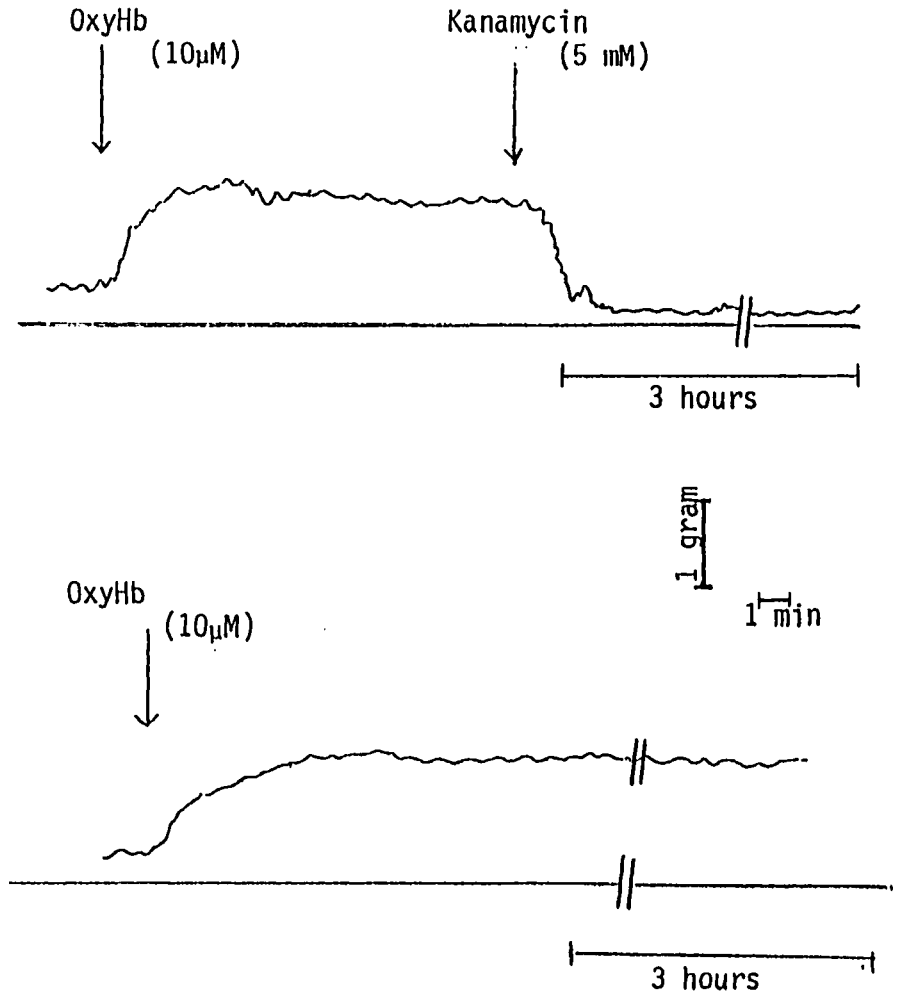


Fig. 15. Representative traces of the effects of kanamycin (5 mM) on canine basilar artery rings preconstricted with OxyHb (10 μM) over a time period of three hours

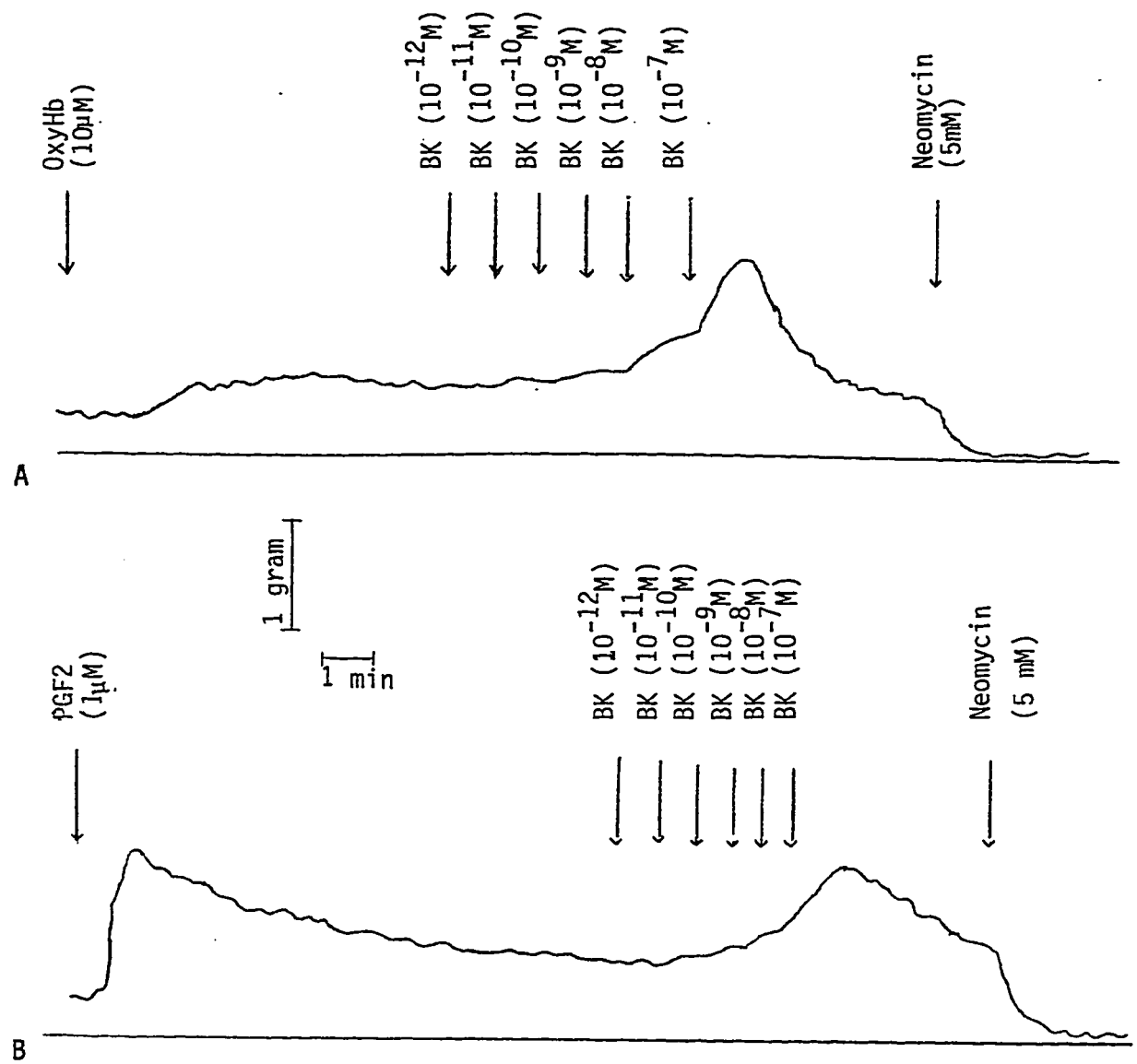


Fig. 16. Representative traces of the effects of removal of endothelium on Neomycin actions. Canine basilar artery rings were preconstricted with OxyHb (10μM) (Fig 14.A) and PGF2α (1μM) (Fig 14.B) bradykinin (10^{-12} -10^{-7} M) were used to confirm the absence of the endothelium. Neomycin (5 mM) was added on top of residual tension.

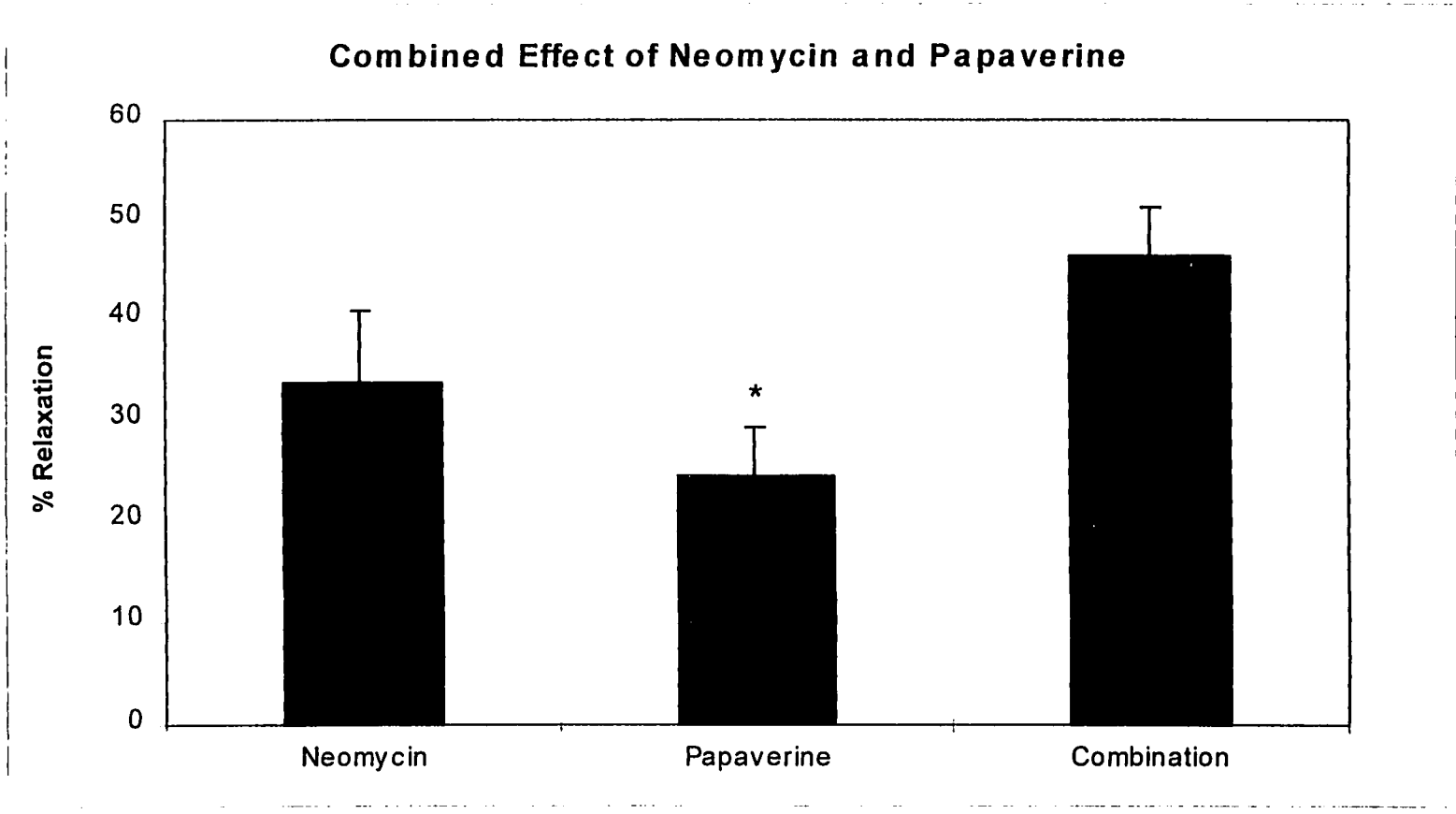


Fig. 17. Comparison of the relaxant effects of submaximal concentrationsof neomycin (1mM), papaverine (1μM) and a combination of both on canine basilar artery rings preconstricted with PGF2α (1μM). Vertical bars indicate SEM (n=5-10). *p<0.05 compared to combination of papaverine and neomycin.

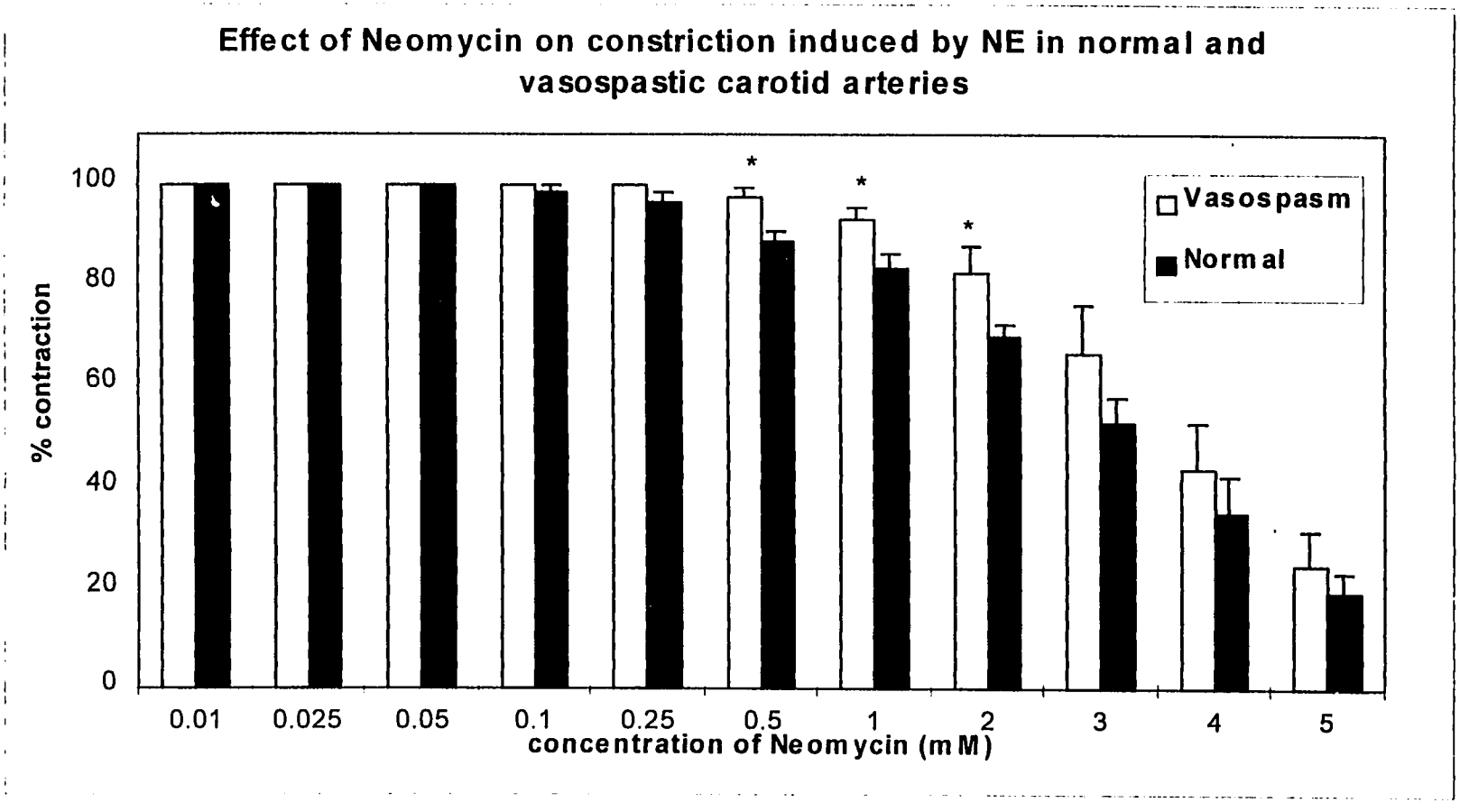


Fig. 18. The vasorelaxant effect of increasing concentrations of neomycin on normal and spastic carotid artery rings obtained from an *in vivo* model of vasospasm. Vertical bars indicate SEM (n=8-9). *p<0.05 compared to control.

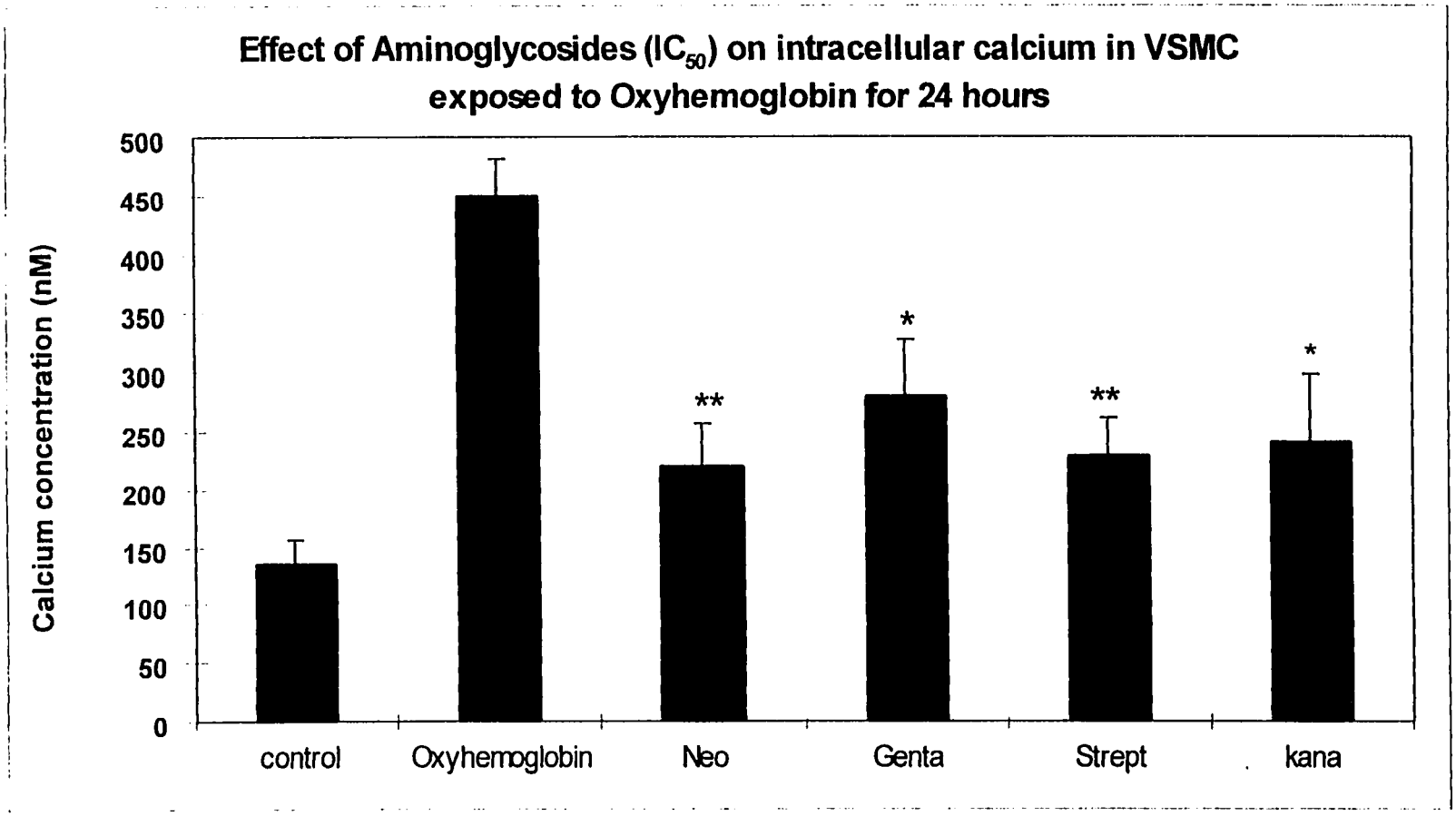


Fig. 19. Effects of aminoglycosides in IC_{50} contraction as determined from contractility studies, against constriction to OxyHb, on intracellular calcium in primate cerebrovascular smooth muscle cells exposed to OxyHb (1μM) for 24 hours. Vertical bars indicate SEM (n=5-7). * $p<0.05$, ** $p<0.01$ compared to OxyHb.

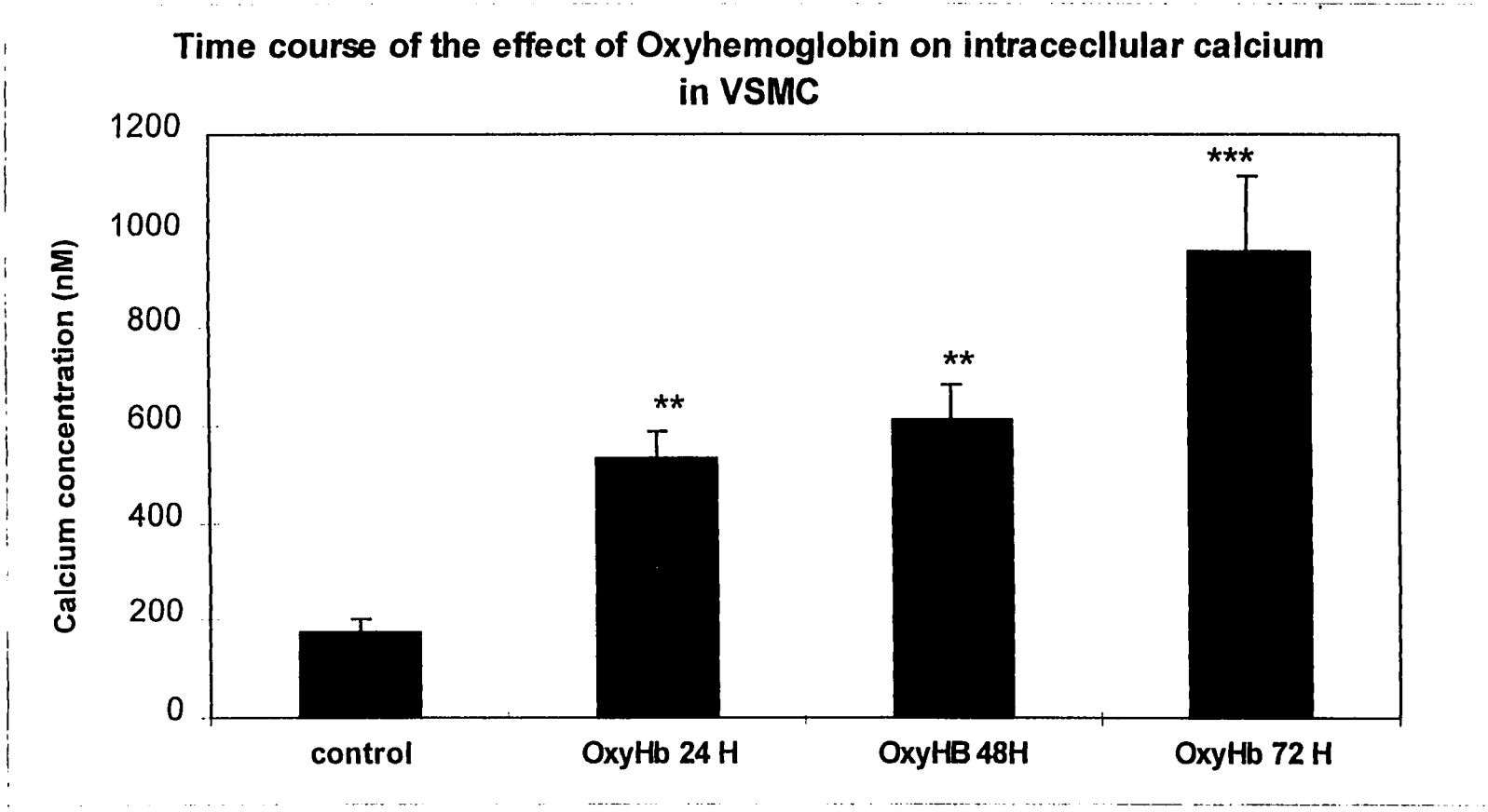


Fig. 20. Intracellular calcium levels in primate cerebrovascular smooth muscle cells exposed to OxyHb (1μM) for 24, 48, and 72 hours. Vertical bars indicate SEM (n=8-25). **p<0.01, ***p<0.001 compared to control.

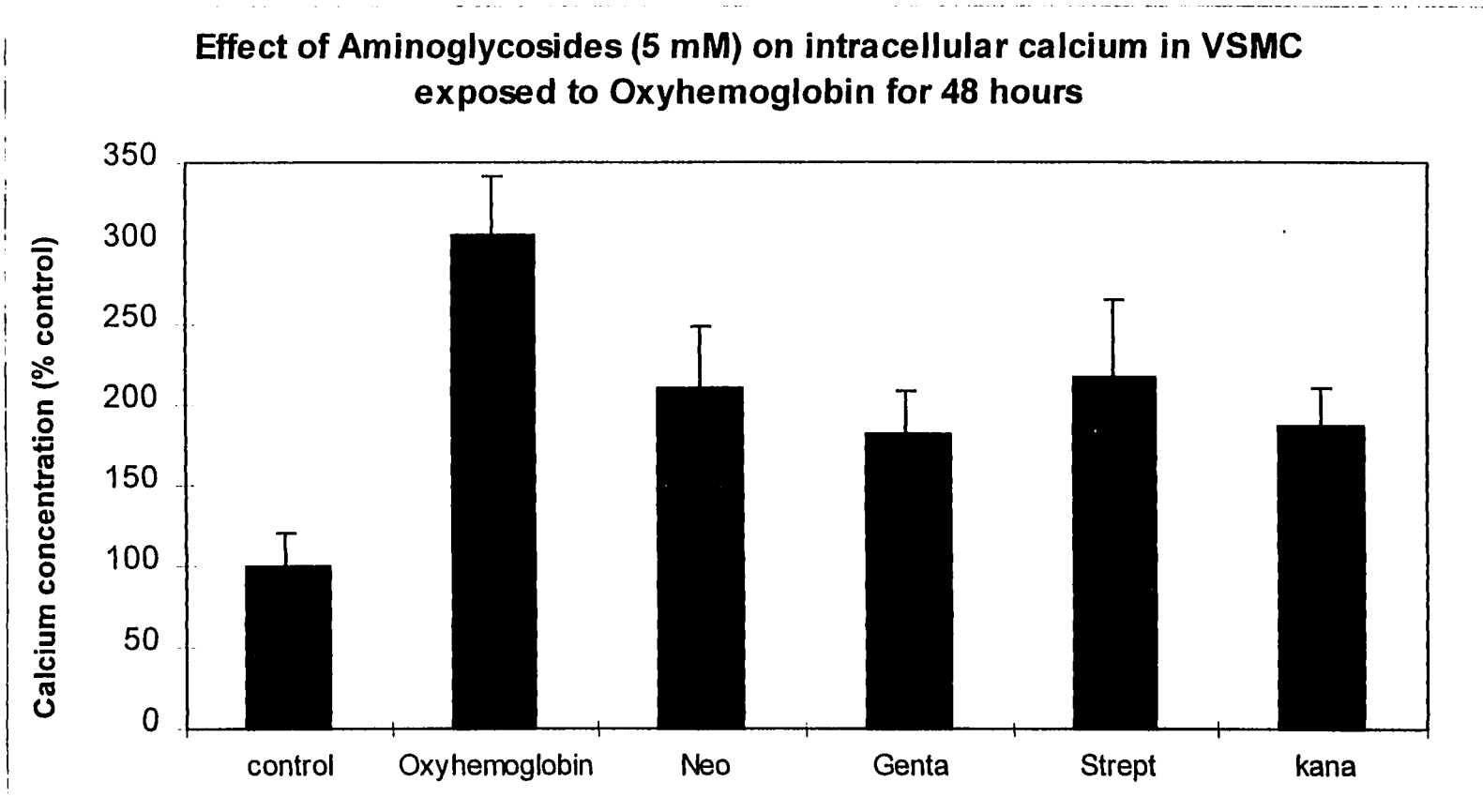


Fig. 21. Effects of aminoglycosides (5mM) on intracellular calcium in primate cerebrovascular smooth muscle cells exposed to OxyHb (1μM) for 48 hours. Vertical bars indicate SEM (n=4-7).

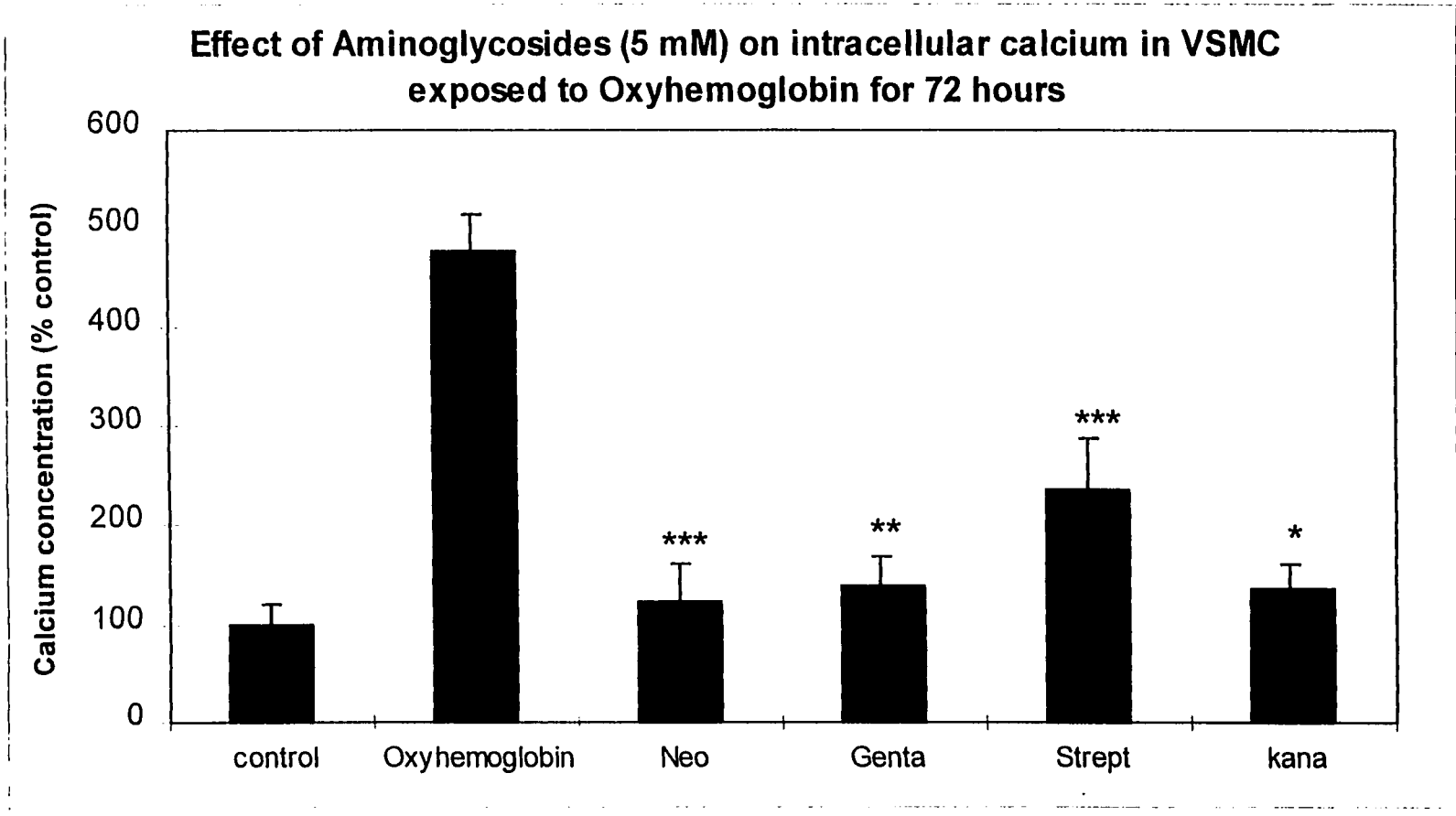


Fig. 22. Effects of aminoglycosides (5mM) on intracellular calcium in primate cerebrovascular smooth muscle cells exposed to OxyHb (1μM) for 72 hours. Vertical bars indicate SEM (n=3-6). *p<0.05,**p<0.01, ***p<0.001 compared to OxyHb.

	Neomycin	Gentamicin	Streptomycin	Kanamycin
OxyHb	0.46±0.10 mM (n=6)	0.53±0.08 mM (n=12)	1.60±0.29 mM (n=7)	3.88±0.46 mM (n=5)
ET-1	0.63±0.08 mM (n=5)	0.54±0.05 mM (n=5)	1.88±0.46 mM (n=3)	2.34±0.92 mM (n=5)
PGF2α	0.89±0.21 mM (n=7)	0.67±0.07 mM (n=5)	0.90±0.09 mM (n=3)	3.72±0.64 mM (n=4)
KCl	0.86±1.4 mM (n=5)	1.12±0.14 mM (n=4)	2.34±0.75 mM (n=5)	3.74±0.55 mM (n=5)
PMA	1.63±0.27 mM (n=6)	1.54±0.34 mM (n=5)	1.43±0.35 mM (n=5)	4.51±0.48 mM (n=4)

Table 1 Estimated $IC_{50's}$ of Aminoglycosides against constrictions induced by different vasoconstrictors

CHAPTER FOUR

DISCUSSION

The pathogenesis of cerebral vasospasm following subarachnoid hemorrhage is multifactorial. There is little doubt that blood products trigger the cascade of events leading to sustained vasospasm. A great body of evidence points to leading roles of OxyHb and endothelin in the pathogenesis of this condition. There is also evidence that the release of vasoactive prostaglandins such as PGF2(may contribute to the development of vasospasm. The signaling events activated by these spasmogens have been shown to involve the activation of phospholipases, intracellular calcium elevation and activation of PKC. As mentioned in the introduction, neomycin has long been known as an inhibitor of PLC activity and subsequent formation of Ins (1,4,5) P_3 formation, a property that is independent of its bactericidal effects, and as such was used to test the involvement of this process in vasospasm. Although this compound probably owes some of its relaxant action to inhibition of PLC, there seem to be additional mechanisms for its inhibitory effects which may be relevant to vasospasm. These studies have demonstrated that many of these actions are shared by other aminoglycosides with characteristic structural features, including gentamicin, streptomycin and kanamycin. These compounds have been shown to be effective in both the reversal of contractile actions of spasmogens investigated, and in the inhibiting of the intracellular calcium accumulation and PKC activity, the key processes which are believed to be involved in the development of cerebral vasospasm. Moreover, the potency of these compounds seem to be related to the number of amino groups each compound possesses.

The effects exerted by these compounds on the intracellular actions mediated by OxyHb, endothelin and PGF2α are discussed below.

I. EFFECTS OF THE AMINOGLYCOSIDE ANTIBIOTICS ON THE MECHANISMS INVOLVED IN SUSTAINED VASOCONSTRICTION

It is well established that the initial phase of smooth muscle contraction is mediated by a rise in intracellular calcium which results in the Ca-CaM dependent activation of MLCK and cross-bridge formation. The sustained tonic response which follows the initial rapid phase is believed to involve extracellular calcium influx and PKC activation. This sustained phase of the response is thought to represent the vasoconstriction which initiates cerebral vasospasm. The present studies have demonstrated that OxyHb produced a slowly developing contraction of isolated canine basilar arteries. The contractile responses of these preparations to endothelin and PGF2α occurred more rapidly but they were also characterized by a sustained phase of the response. The contractile activity of OxyHb is thought to be initiated by the generation of free radicals with subsequent lipid peroxidation leading to the activation of phospholipases (Cook and Vollrath, 1995). This proposal has been supported by the observations that OxyHb, hydrogen peroxide and lipid peroxides increase Ins (1,4,5) P_3 formation and intracellular calcium release in cultured smooth muscle cells (Vollrath *et al.*, 1990). These observations suggest that at least part of the action of OxyHb is mediated by PLC. In the present studies the PLC inhibitor, neomycin, attenuated the contractile responses to OxyHb, thus supporting the proposal that activation of PLC may play a role in the vasoconstriction produced by OxyHb. The effect of neomycin on the responses of cerebral arteries to OxyHb is particularly interesting because the contractile action of OxyHb is known to be resistant to many vasodilators including prostaglandin synthesis inhibitors, and receptor antagonists (Macdonald and Weir, 1991 ; Tanishima,

1980). Neomycin has been shown to bind selectively to phosphoinositides thus interfering with the enzymatic hydrolysis of these lipids mediated by PLC. This interaction of neomycin with phosphatidylinositol (4,5) bis phosphate has provided the basis for the use of this agent as a tool to study the possible involvement of PLC and Ins (1,4,5) P_3 in intracellular processes. For example, preincubation with neomycin has been shown to reduce the OxyHb-induced increase in Ins (1,4,5)P_3 formation by 50%, an observation which is consistent with the involvement of PLC in the action of OxyHb (Vollrath *et al.*, 1990).

In the present studies, in addition to inhibition of OxyHb-mediated responses, neomycin also inhibited the contractile responses to endothelin and PGF2α, the agents whose activity is mediated by receptor-mediated activation of PLC. The receptors for these agents are coupled to Gq protein, which is known to stimulate PLC activity and subsequent Ins (1,4,5) P_3 formation and intracellular calcium release. Neomycin was also shown to relax the contractions mediated by PGF2α in calcium free medium, an effect which is characteristic for PLC and Ins (1,4,5) P_3-mediated responses. All these observations support the proposal that PLC activation is involved in the initiation of vasoconstriction by the spasmogens. The inhibitory effects of neomycin on vasoconstriction induced by OxyHb, endothelin and PGF2α were shared by other aminoglycosides, including gentamicin, kanamycin and streptomycin. These compounds differ in the chemical nature and number of positive charges, having five, four and two amino groups, respectively. There was a good correlation between the inhibitory effects of the individual aminoglycosides and the number of amino groups, thus indicating that the relaxant effects produced by these agents are dependent on their structure. Of the examined aminoglycosides neomycin and gentamicin appeared to be the most potent with the $IC_{50's}$ of about 0.5 mM. The similarities in the order of potency of the aminoglycosides against sustained vasoconstriction induced by OxyHb, endothelin-1 and PGF2α, suggest that the effects of these agents are mediated by the inhibition of the

mechanisms common for all the spasmogens. The involvement of some of the putative mechanisms in sustained vasoconstriction is discussed below.

Neomycin and other aminoglycosides also inhibited vasoconstriction to the spasmogens a long time after tonic response had been developed, thus indicating that these agents may have additional sites of action, because the activation of PLC and Ins (1,4,5) P_3 production is transient. It is well documented that within about 5 minutes after stimulation, the levels of Ins (1,4,5)P_3 are not different from the controls (Vollrath *et al.*, 1990). These observations indicate that the sustained responses to OxyHb, endothelin and PGF2α are unlikely to arise from activation of PLC alone and that mechanism(s) in addition to inhibition of PLC may be involved in the vasorelaxant action of aminoglycosides. This suggestion is further supported by the observation that the aminoglycosides inhibited vasoconstriction to high depolarizing concentrations of potassium chloride which produces contraction through the stimulation of calcium influx via VDCC, a process independent of PLC activation. Although VDCC do not seem to play a major role in experimental vasospasm or in OxyHb-induced vasoconstriction, the observation that aminoglycoside antibiotics inhibit these channels is interesting because it may provide an additional mechanism by which aminoglycosides inhibit endothelin-mediated vasoconstriction. The signaling events mediated by the endothelin receptors are complex and include PLC and PKC activation and calcium influx mediated by both voltage dependent and receptor dependent calcium channels (Haynes and Webb, 1993 ; Hirata *et al.*, 1988). Thus, the ability of aminoglycosides to relax endothelin-mediated vasoconstriction is probably due not only to the inhibition of PLC and PKC activity but also to the inhibition of the voltage and receptor dependent calcium channels.

The observation that OxyHb is involved in cerebral vasospasm suggests that the effects mediated by endothelium might also be involved, because the haem moiety of OxyHb is known to bind nitric oxide, a diffusable vasodilator

synthesized in endothelial cells, thus preventing its relaxant effects in vascular smooth muscle cells (Hongo *et al.*,1988). This mechanism was proposed to account, in part, for the vasoconstricting effect of OxyHb. In this study, the removal of vascular endothelium resulted in the inhibition of bradykinin mediated vasodilatation, however, it did not affect the vasorelaxant actions of neomycin, thus indicating that the effects of this agent on vasoconstriction mediated by OxyHb are endothelium independent. Because other aminoglycosides have similar structural features to these represented by neomycin, it is likely that their vasorelaxant effects are also endothelium independent. Furthermore, these studies have provided evidence that OxyHb-mediated contraction is endothelium independent.

Another important finding made in these studies is the observation that the relaxant effects of aminoglycosides were maintained over long period of time. The contractile effects of all the spasmogens examined are sustained, in particular these mediated by OxyHb, and it is conceivable that inhibition or reversal of these effects require administration of drugs with a long term vasorelaxant action.

1. EFFECTS OF AMINOGLYCOSIDES ON CONSTRICTIONS INDUCED BY THE ACTIVATION OF PKC

Cerebrovascular spasm results in a prolonged elevation of DAG which causes persistent activation of PKC (Matsui *et al.*, 1991). There is evidence indicating that the sustained activation of PKC is an important step in the mechanisms of actions of endothelin and OxyHb. Pretreatment with staurosporin, H7 or chelerythrine, well known inhibitors of PKC, was shown to reduce the contraction to OxyHb. More importantly, these inhibitors were shown to produce a concentration-dependent inhibition of responses to OxyHb when

given at the peak of tension, thus indicating the involvement of PKC in sustained contraction produced by OxyHb (Cook *et al.*, 1993).

Tumor promoting phorbol esters were used in the present study to constrict arterial preparations and to investigate the effects of aminoglycosides on PKC-induced constriction. Both phorbol esters and DAG are known to bind to specific cysteine residues present in the regulatory subunit of PKC, thus selectively activating this enzyme (Gschwendt *et al.*,1991). Phorbol esters produced a slowly developing sustained constriction that was difficult to wash out. The aminoglycosides inhibited these constrictions in a concentration dependent manner. Neomycin and gentamicin produced relaxation which was comparable to that induced by specific PKC inhibitors. These observations confirm previous findings which demonstrated that aminoglycosides and other polyamine compounds are effective inhibitors of purified preparations of PKC isolated from kidney and brain (Hagiwara *et al.*, 1988). It is rather unlikely that aminoglycosides inhibit the enzyme by direct interaction with the enzyme because PKC is an intracellular enzyme, and would thus not be accessible to aminoglycosides which do not permeate plasma membrane. One possible mechanism by which aminoglycosides may inhibit PKC is the interaction of these agents with phosphatidylserine. The process of activation of PKC is associated with translocation of this enzyme from the cytosol to the plasma membrane where it binds phosphatidylserine, a specific cofactor required for the PKC activation. This interaction promotes a conformational change in the enzyme which allows its activation by DAG or phorbol esters (Nishizuka, 1995). A proposed explanation for the inhibitory action of aminoglycosides on PKC activity is that positively charged aminoglycosides bind to phosphatidylserine, which is an acidic phospholipid, and thus inhibit the interaction of PKC with DAG in the membrane and consequently block the vasoconstriction. The observation that aminoglycosides inhibit constrictions mediated by PKC activation represents a novel finding and since most of the spasmogens implicated in vasospasm were

shown to stimulate PKC activation, this finding may be of importance in the study of vasospasm.

2. EFFECTS OF AMINOGLYCOSIDES ON INTRACELLULAR CALCIUM

Earlier work from this laboratory and elsewhere has shown that OxyHb produces a sustained elevation of intracellular calcium in smooth muscle cells which may be responsible for the sustained vasoconstriction of cerebral arteries. The increase in calcium level was initiated by OxyHb about 1 minute after exposure of the vascular smooth muscle cells to this agent, and maintained for up to 7 days (Takanashi *et al.*, 1992). It has also been shown that this action of OxyHb can be prevented or reversed by neomycin (Vollrath *et al.*, 1994). In the present study we attempted to examine whether other aminoglycoside antibiotics may inhibit the accumulation of intracellular calcium induced by prolonged exposure of the cells to OxyHb.

Administration of OxyHb to cerebrovascular smooth muscle cells caused an elevation of intracellular calcium which was maintained and even increased over time. The calcium level increase was about 300% after 24 hours and about 500% after 72 hours. These results are consistent with previous reports which have shown that long term exposure to OxyHb. resulted in the accumulation of intracellular calcium (Vollrath *et al.*, 1994).

All the tested aminoglycosides effectively inhibited the cytosolic calcium elevation observed after exposure to OxyHb. for 24 hours. In the present experiments the aminoglycosides were administered three hours after initial exposure to OxyHb. This approach has some practical implications. There is a lag period between the hemorrhage and the occurrence of vasospasm that allows a therapeutic intervention. For an agent to be of practical value in vasospasm, it has to be effective when administered before and after

development of vasospasm. In an attempt to mimic clinical vasospasm we extended the study period to three days. In cells exposed to neomycin, gentamicin, streptomycin and kanamycin, the increase in calcium was reduced to levels not different from control in each case. These results demonstrate that the aminoglycosides may be effective in inhibiting the intracellular reactions already initiated by OxyHb present in the culture medium.

We also attempted to determine whether the rank order of potency of aminoglycosides in inhibiting intracellular calcium accumulation parallels their ability to produce relaxation of contractions induced by OxyHb. These experiments have shown that the aminoglycosides administered in half maximum concentrations, derived from contractility studies, inhibited intracellular calcium by about 50%, thus indicating that the rank order of these agents in both reducing calcium accumulation and producing relaxation of cerebral vessels contracted by OxyHb is very similar. In summary, the present study has provided evidence that the aminoglycosides are effective inhibitors of intracellular calcium accumulation induced by OxyHb over a long period of time, and there is a relation between the effectiveness of these compounds and the number of positive charges each compound possesses.

The mechanism(s) by which aminoglycosides reduce calcium accumulation stimulated by OxyHb is unclear. One possible mechanism would be through the inhibition of PLC activity. Neomycin has been known to inhibit PLC-catalyzed phosphoinositides hydrolysis and the present studies have provided evidence that the ability of this compound to inhibit PLC is shared by other aminoglycosides. However, the Ins (1,4,5) P_3 elevation by OxyHb and endothelin is transient and thus it is unlikely to account for a sustained intracellular calcium elevation. It is conceivable that PLC activation and subsequent formation of Ins(1,4,5)P_3 is responsible only for the triggering of intracellular calcium release and that it is this initial rise in calcium which is inhibited by the aminoglycosides via inhibition of phosphoinositides hydrolysis. Another possible mechanism which might contribute to the sustained calcium

elevation stimulated by OxyHb, and which can be inhibited by aminoglycosides, is calcium influx across cell membrane. The type of channels responsible for calcium influx stimulated by OxyHb is obscure but it is unlikely to be VDCC of L-type. As mentioned earlier, the responses to OxyHb are resistant to the actions of the dihydropyridine-derivatives, well known antagonists of the L-type calcium channels, thus indicating that calcium influx is probably mediated by mechanism other than voltage-dependent calcium entry (Takenaka *et al.*, 1991). Antagonistic interactions between calcium and aminoglycosides have been described by several authors. Neomycin was shown to decrease total uptake of radioactive calcium and to increase calcium efflux in rabbit aortic smooth muscle on the basis of these results, it was proposed that neomycin might alter the responsiveness of vascular smooth muscle to many vasoactive agents. The mechanism for the inhibitory actions of neomycin on calcium channels is unknown (Adams *et al.*, 1974). The mechanism for the antagonistic interactions between neomycin and Ca^{2+} is not clear. Neomycin, like other aminoglycosides is a polycataionic organic base with several functional amino groups. Molecular events leading to uptake and binding of Ca^{2+} are thought to involve association of Ca^{2+} with anionic binding sites localized in plasma membrane calcium channel proteins (Catarell and Striessnig, 1992 ; Schwartz, 1994). It is conceivable that there is a charge interaction between aminoglycosides and acidic residues in the channel pores. This interaction leads to inhibition of calcium entry. An explanation for the sustained calcium elevation stimulated by OxyHb may be the disturbance in calcium homeostasis which may result from damage of the Ca-ATPase pump by free radicals generated by OxyHb. Normally, intracellular calcium levels are maintained at low, nanomolar levels over a broad range of concentrations by the action of calcium transporters and calcium pumps present in plasma, endoplasmic reticulum and mitochondrial membranes. Large accumulation of calcium observed over long period of time suggest that the mechanisms responsible for calcium homeostasis may be impaired by OxyHb.

There is evidence that the function of the plasma membrane calcium pump is impaired in experimental vasospasm (Wang *et al.*, 1994). This observation is consistent with the finding that the calcium pump in erythrocytes is inhibited by free radicals, an action which can be prevented by 21-aminosteroids (Rhon *et al.*, 1996). This suggests that free radicals derived in the process of oxidation of OxyHb might cause calcium pump damage which can result in the inhibition of calcium extrusion and subsequent accumulation of intracellular calcium. There is also evidence that calcium pump can be proteolytically damaged by calpain, a protease activated by elevated calcium levels (Minami *et al.*, 1992). The polyamines, spermine and spermidine, have been reported to have protective effects against free radical toxicity and lipid peroxidation in plasma membrane (Chapman and Wallace, 1994). If such properties are shared by polyamine antibiotics this could provide an explanation for their ability to inhibit intracellular calcium accumulation induced by OxyHb.

II. STRUCTURE ACTIVITY RELATION OF AMINOGLYCOSIDES

If the actions of aminoglycosides were due to their cationic nature then it would be expected that order of potency of these agents is proportional to the number of positive charges they carry. In the present study, the general trend in potency of the aminoglycosides in inhibiting vasoconstriction, estimated on the basis of their $IC_{50's}$ against different agonists, was : neomycin>gentamicin> streptomycin > kanamycin. This order of potency reflects the number of positive charges in individual aminoglycosides: neomycin and gentamicin, which have 6 and 5 ionizable amino groups, were most potent, while kanamycin which has 4 positive charges was less effective. While streptomycin has only 2 ionizable amino groups it has different aminocyclitol ring which may account for the deviation observed with this agent. The aminocyclitol ring in streptomycin is

streptidine, while deoxystreptamine is common to the other three aminoglycosides (Lortholary *et al.*, 1995). Similar order of potency was observed in the experiments examining the effects of aminoglycosides in the KCl-induced contractions and intracellular calcium levels, thus indicating that the ability of aminoglycosides to interfere with multiple cellular processes is related to their structure.

The relation between the number of positive charges and the effects of aminoglycosides has been investigated by many groups using a variety of tissues and different experimental approaches (Hagiwara *et al.*,1988 ; Hostetler and Hall, 1982). Schacht was the first to suggest the presence of the relation between positive charges carried by aminoglycosides under physiological pH and the effects of these agents on phosphoinositide hydrolysis. He also showed that naturally occurring polyamines share the ability of aminoglycosides to bind to phosphoinositides (Schacht, 1976). Hagiwara *et al.*(1988) studied the effects of aminoglycosides on PKC activity and reported a linear correlation between the number of amino groups in aminoglycosides and their potency in inhibition of this enzyme. Streptomycin was reported in many studies to possess slightly different properties than the other aminoglycosides, an observation that may be attributed to the presence of streptidine. Our studies have provided evidence that the potency of aminoglycosides in inhibiting many different processes, including actions on PLC, PKC, VDCC and intracellular calcium accumulation, is related to the structure of these compounds. However, to prove conclusively such a relationship a wider spectrum of polycationic compounds may need to be examined. An understanding of the relation between the structure and the functional properties may help to develop new compounds with higher potency and hopefully less toxicity.

III. EFFECTS OF NEOMYCIN ON VASOSPASTIC VESSELS

The results presented here indicate that neomycin produced rapid and effective relaxation of the tonic contractions, induced by noradrenaline in the canine high-cervical carotid artery, obtained from animals with vasospasm that has been confirmed angiographically. The responses of vasospastic vessels to noradrenaline were significantly attenuated in comparison to the responses of normal arteries. This observation is consistent with earlier studies which have demonstrated reduced responsiveness of cerebral blood vessels to many vasoconstrictors after cerebral vasospasm. Functional changes of primate cerebral arteries in chronic cerebral vasospasm included reduced distensibility, a significant increase in arterial muscle tone, and a decreased ability of arteries to contract (Bevan *et al.*, 1987). More recent studies have reported a decreased responsivity of human cerebral arteries after SAH to several agonists including noradrenaline, serotonin and PGF2α (Onoue *et al.*, 1995). The mechanism responsible for the diminished contractility is unclear. However, it is likely that smooth muscle cells can only contract to a limited extent and that they are already contracted in vasospasm. Reduced responsiveness of cerebral vessels after SAH to vasorelaxants such as bradykinin, acetylcholine and calcium ionophore, A23187, has also been reported (Bevan *et al.*, 1987), thus indicating that endothelial cells function may be impaired in vessels after SAH. Our present observation that neomycin produces relaxation of spastic arteries that is almost indistinguishable from relaxation induced in control arteries, suggests that this agent acts via mechanism that is not significantly affected by blood. Thus these experiments indicate that neomycin, and probably other aminoglycosides, are effective vasodilators of vessels in spasm.

IV. CONCLUSIONS

The present study provided evidence that :

- The polycationic aminoglycoside antibiotics are effective inhibitors of constrictions induced by major spasmogens implicated in vasospasm.
- The aminoglycosides seem to have multiple mechanisms of actions including inhibition of both PLC and PKC as well as inhibitory effects on calcium influx.
- The aminoglycosides are effective inhibitors of intracellular calcium elevation induced by OxyHb over prolonged periods of time.
- The actions of aminoglycosides seem to be related to their polycationic structure.
- The aminoglycosides or compounds with similar chemical structures may be of potential benefit in the management of cerebral vasospasm.

CHAPTER FIVE

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Abstracts

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6. Gergawy M., Vollrath B. and Cook D. Aminoglycosides inhibit the action of oxyhemoglobin and endothelin-1 in basilar artery. Proceedings of the 6th international conference on cerebral vasospasm. (accepted).

Papers

1. Gergawy M., Vollrath B. and Cook D. Effects of aminoglycoside antibiotics on cerebral vasospasm following subarachnoid hemorrhage. (in preparation).