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The mechanism by which aminoglycoside antibiotics cause vasodilation of canine cerebral arteries

[1]Mourad Gergawy, [1,2]Bozena Vollrath & [1]David Cook

[1]Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

1 The effects of aminoglycoside antibiotics were examined in canine cerebral arteries and in cultured cerebrovascular smooth muscle cells stimulated with oxyhemoglobin (OxyHb), a blood constituent which has been implicated in the pathogenesis of cerebrovascular spasm.

2 In cerebral arterial rings precontracted with OxyHb (10 μM), the aminoglycosides caused a concentration-dependent decrease in isometric tension. The EC_{50}s for the relaxation were 0.46 ± 0.1 mM ($n=6$), 0.53 ± 0.08 mM ($n=12$), 1.6 ± 0.3 mM ($n=7$) and 3.9 ± 0.5 mM ($n=5$) for neomycin, gentamicin, streptomycin and kanamycin, respectively. This order of potency corresponds approximately to the number of positive charges in the molecules.

3 The aminoglycosides also inhibited the contractions to prostaglandin $F_{2\alpha}$ (1 μM) and depolarizing concentrations of potassium chloride (60 mM). The order of potency was neomycin > gentamicin > streptomycin > kanamycin.

4 The relaxation was maintained in vascular preparations denuded of endothelium.

5 Neomycin (5 mM) abolished the Ca^{2+}-independent contraction to $PGF_{2\alpha}$.

6 In Fura-2-loaded cerebrovascular smooth muscle cells, OxyHb (1 μM) significantly enhanced the concentration of intracellular calcium ($[Ca^{2+}]_i$) by 330%. The administration of neomycin, gentamicin, kanamycin and streptomycin in concentrations corresponding to the EC_{50} from contractility studies, reduced the effects of OxyHb on $[Ca^{2+}]_i$ by about 50% to 221 ± 35 nM ($n=7$), 270 ± 31 nM ($n=7$), 229 ± 33 nM ($n=6$) and 240 ± 6 nM ($n=5$), respectively.

7 These results suggests that the effects of the aminoglycosides on the OxyHb-induced contraction and the long-term increase in $[Ca^{2+}]_i$, may arise from several effects, including inhibition of PLC, protection of calcium extrusion mechanisms, and interference with the process of $[Ca^{2+}]_i$ accumulation.

Keywords: Cerebrovascular spasm; cerebral arteries; oxyhemoglobin; prostaglandin $F_{2\alpha}$; aminoglycoside antibiotics; intracellular calcium

Introduction

Cerebrovascular spasm is a sustained constriction of the cerebral arteries which develops several days after subarachnoid haemorrhage (SAH) (Findlay *et al*., 1991). The condition is the leading cause of morbidity and mortality in patients who have recovered from the initial haemorrhage, but at present no agents have been shown to reverse or to inhibit vasospasm once it has begun. There is evidence from our laboratory and elsewhere which suggests that oxyhaemoglobin (OxyHb) is the causative factor and that the delayed arterial spasm is probably a consequence of the lysis of erythrocytes in the clot causing release of OxyHb (Harada *et al*., 1990; Vollrath *et al*., 1994). OxyHb is known to cause sustained smooth muscle contraction in isolated vascular preparations, and there is convincing evidence that the clinical condition arises from this process (Macdonald *et al*., 1991). While the involvement of OxyHb in the pathogenesis of vasospasm is difficult to dispute, the mechanism by which this agent causes vasospasm is still far from clear. One hypothesis proposes that generation of oxygen free radicals in the process of oxidation of OxyHb to methemoglobin, and the subsequent lipid peroxidation, represent the initial step in the development of vasospasm (Macdonald & Weir, 1994). The free radicals and lipids peroxides have been shown to stimulate a variety of cellular processes including activation of phospholipases C (PLC), A_2 and D, protein kinase C (PKC) and mitogen activated protein kinase (MAPK), as well as the expression of proto-oncogenes (Abate *et al*., 1990; Cook & Vollrath, 1995; Guyton *et al*., 1996). We have shown that in vascular smooth muscle cells, OxyHb produces a transient increase in inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) formation, an observation which suggests that the hydrolysis of phosphatidylinositol (4,5) bis-phosphate by PLC may be an early step in the process (Vollrath *et al*., 1990). We have also shown that OxyHb causes an increase in the level of intracellular calcium ($[Ca^{2+}]_i$) with a time-course which parallels the contractile action of OxyHb, suggesting that these events might be functionally linked (Vollrath *et al*., 1994).

The increase in Ins(1,4,5)P_3 and the vasoconstriction caused by OxyHb are both inhibited by neomycin, an aminoglycoside antibiotic for Gram-negative bacterial infections and a nonselective inhibitor of phospholipase C (PLC). This agent binds to inositol phospholipids and thus prevents the hydrolysis of these lipids by PLC (Schacht, 1978). Neomycin and the related aminoglycoside antibiotics have been widely used to study the involvement of this class of lipids in a variety of cellular processes including smooth muscle contraction (Adams *et al*., 1974; Cockcroft & Gomperts, 1985; Streb *et al*., 1985; Prentki *et al*., 1986;). In addition to their action on inositol phospholipids, aminoglycoside antibiotics have been shown to inhibit PKC (Hagiwara *et al*., 1988), transmembrane calcium channels (Keith *et al*., 1992), and calcium release from the sarcoplasmic reticulum of skeletal muscle (Palade, 1987), a system in which Ins(1,4,5)P_3 is ineffective as a second

[2]Author for correspondence.

messenger. This last observation suggests that the inhibitory effect of the aminoglycosides on smooth muscle contraction may be mediated by mechanisms other than those involved in the inositide pathway. The aminoglycoside antibiotics are calcium antagonists in several types of peripheral blood vessels, as well as in the myocardium, autonomic ganglia, kidney and neuromuscular junction (Corrado, 1958; Wolf & Wigton, 1971; Miao & Lee, 1991; Godicke *et al*., 1992; Pichler *et al*., 1996). Furthermore, the results of studies *in vivo* using a primate model of cerebral vasospasm (Zervas *et al*., 1974) and clinical trials in humans (Zervas *et al*., 1979), suggested that the aminoglycoside antibiotic kanamycin, had some beneficial effects in angiographic spasm. At that time, the intracellular mechanisms involved in the pathogenesis of vasospasm were largely unknown; kanamycin was tested on the basis of a hypothesis which was rapidly rejected and thus these reports have passed largely unnoticed.

All these observations suggest that neomycin and related aminoglycosides may be effective in inhibiting the cellular processes thought to be involved in the pathogenesis of cerebral vasospasm. We have thus investigated the effects of neomycin and other structurally related aminoglycosides on the vasoconstriction induced by OxyHb in canine cerebral arteries. Because of the pivotal role of changes in the level of intracellular calcium in the development of vasospasm, the effects of the aminoglycoside antibiotics on intracellular calcium levels have also been measured in cerebrovascular smooth muscle cells treated with OxyHb.

Methods

Recording of isometric tension

Mongrel dogs of either sex, weighing about 20 kg, were used in these studies. Protocols for the humane treatment of animals, according to the Declaration of Helsinki, and as approved by the University of Alberta Animal and Ethics review Committee were followed in all experiments. The animals were killed with an intravenous (i.v.) overdose of pentobarbitone, and the brain with the cerebral arteries attached was removed and placed in oxygenated Krebs-Henseleit solution of the following composition (in mM): Na^+ 130, K^+ 5, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 12.2, HCO^{3-} 25, SO_4^{2-} 1.2, $H_2PO_4^{2-}$ 1.2 and dextrose 11. The middle portion of the basilar artery was cut into sections about 3 mm in length and these preparations, either intact or with the endothelium removed mechanically, were suspended in tissue baths containing Krebs-Henseleit solution at 37°C, gassed with 95% O_2 and 5% CO_2. The arterial rings were equilibrated for 1 h. Contractions to OxyHb were recorded isometrically using force-displacement transducers and a Grass 7D polygraph. OxyHb produced a contraction which reached a maximum, defined as 100% at times which varied from 4 to 15 min after the agent was first administered. The presence of an intact endothelium in an arterial ring preparation was examined by evaluating the relaxant responses to bradykinin, in preparations which had been pre-contracted with 1 μM prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$).

Rings obtained from basilar arteries from which the endothelium had been removed, were preconstricted with OxyHb (10 μM), $PGF_{2\alpha}$ (1 μM) or with high concentrations of potassium chloride (60 mM). The preparations were then exposed to the aminoglycoside antibiotics administered in increasing, cumulative concentrations (0.01 – 5 mM) to preparations in which a tonic contraction to OxyHb had developed. In order to determine whether the relaxation produced by the aminoglycosides were persistent or transient, the responses were recorded for at least 3 h. The relaxation of the preparations exposed to the aminoglycoside antibiotics was expressed as the percentage of maximum relaxation produced by 0.5 mM papaverine chloride. EC_{50} values for the aminoglycoside antibiotics were determined using a nonlinear regression analysis.

Cerebrovascular smooth muscle cell culture

Cells were prepared as described previously (Vollrath *et al*., 1994). Briefly, basilar arteries were isolated under sterile conditions as described above and the middle sections of the arteries were placed in a Petri dish containing Dulbecco's modified Eagle medium (DMEM). The adventitia was removed mechanically, the vessels were cut into segments approximately 5 mm in length along the longitudinal axis, and the endothelium was removed by gently scraping the inner surface of the segments. The tissue was then chopped into 1 – 2 mm pieces and the explants were transferred to 25 cm^2 culture flasks containing 1 ml of DMEM supplemented with 10% foetal calf serum, penicillin (100 u ml^{-1}), and streptomycin (100 μg ml^{-1}). After the explants had adhered, the volume was made up gradually over the next 4 days to 4 ml per flask, and thereafter the culture medium was changed weekly. When the primary cultures were almost confluent the cerebrovascular smooth muscle cells were transferred to 75 cm^2 flasks and then routinely subcultured at a split ratio of 1 : 3.

Determination of intracellular free Ca^{2+}

The concentration of intracellular calcium was determined in suspensions of smooth muscle cells using the fluorescent indicator Fura-2, as described previously (Vollrath *et al*., 1995). In brief, intracellular loading of Fura-2 was carried out by incubation of the monkey or dog cerebrovascular smooth muscle cells for 45 min at 37°C in a modified Krebs-Henseleit buffer containing 5 μM of Fura-2 AM, the acetoxymethylester of Fura-2. The loaded cells were then washed twice and resuspended in fresh buffer at a density of about 10^6 cells ml^{-1}. Measurements were made using a Perkin-Elmer MKF-4 spectrofluorimeter. Fluorescence was measured at excitation wavelengths of 340 and 380 nm and an emission wavelength of 505 nm. Calibration of the resulting signal and calculation of intracellular calcium ($[Ca^{2+}]_i$) were conducted according to the method of Grynkiewicz *et al*. (1985) where R_{min} and R_{max} were determined by treatment with Triton X-100 (0.1%) in the presence of 5 mM EGTA and calcium chloride (5 mM), respectively. These studies were conducted in control cells treated with a vehicle and those exposed to OxyHb and the aminoglycosides for 24 h. The aminoglycosides were added to the cells, at a concentration equal to the EC_{50} values determined from the contractility studies, 3 h after the addition of OxyHb, since this would be expected to simulate more closely the clinical situation, in which therapeutic agents would be administered some time after vasospasm had developed.

Chemicals and other reagents

Human haemoglobin (Hb), $PGF_{2\alpha}$ and sulphate salts of neomycin, gentamicin, kanamycin and streptomycin were obtained from Sigma. Sodium sulphate (15 mM) was used as a vehicle control for the aminoglycosides and was shown to be devoid of any effect on the muscle tension and the fluorescence

ratio. Preparation of OxyHb from human Hb was performed according to the method of Martin *et al.* (1985). The concentration of OxyHb was determined by absorption spectrophotometry. Other chemicals were commercial products of the highest grade available.

Statistical analysis of results

Data are expressed as the mean ± the standard error of the mean, with number of preparations used in parentheses. Statistical significance was assessed using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test when significant probability was reached. Values of $P<0.05$ were considered to be significant.

Results

Effects of the aminoglycoside antibiotics on contractions induced by OxyHb, $PGF_{2\alpha}$ and KCl

OxyHb administered at a single dose of 10 μM, induced a slowly developing sustained contraction. Cumulative concentrations of neomycin abolished contraction (100% relaxation, $n=6$), while the maximal relaxing effects of gentamicin and streptomycin, administered in the same concentrations were 96.8 ± 1.86% ($n=12$) and 86.15% ± 3% ($n=7$), respectively. Kanamycin was the least effective agent and caused a maximum relaxation of 63.4 ± 5.61% ($n=5$). These results are shown in Figure 1. The EC_{50} values for different aminoglycosides against contractions produced by OxyHb are shown in Table 1. The order of potency of the aminoglycosides was neomycin (0.46 ± 0.1 mM) > gentamicin (0.53 ± 0.08 mM) > streptomycin (1.6 ± 0.29 mM) > kanamycin (3.88 ± 0.46 mM).

The aminoglycosides were also very effective against the contraction produced by $PGF_{2\alpha}$, an agonist whose responses are mediated by G-protein coupled receptors. The cumulative concentration-effect curves for neomycin, gentamicin, streptomycin and gentamicin are shown in Figure 2. The contractile responses of cerebral artery preparations to $PGF_{2\alpha}$ occurred more rapidly than those to OxyHb reaching a maximum at about 30 s. Maximal relaxations for neomycin, gentamicin, streptomycin and kanamycin were 96.6 ± 3.1% ($n=6$), 94.1 ± 2.5% ($n=7$), 84.7 ± 6% ($n=3$) and 59.4 ± 0.21% ($n=4$), respectively. The EC_{50} values for these aminoglycosides are shown in Table 1 and show a similar order of potency to that observed when contractions were elicited with OxyHb. The relaxant effects of the aminoglycosides were still observed when the contractile responses to $PGF_{2\alpha}$ were induced in Ca^{2+}-free medium thus suggesting that, at least in part, action of aminoglycosides is mediated by the inhibition of PLC activity. In these experiments, the basilar artery preparations were equilibrated in normal Krebs-Henseleit medium, and the medium was then replaced with Ca^{2+}-free Krebs-Henseleit buffer, containing 2 mM EGTA. These preparations were unresponsive to KCl, but developed a contraction to $PGF_{2\alpha}$. Neomycin produced a marked relaxation of that response, and a further relaxation was induced by papaverine. Representative traces of the effects of $PGF_{2\alpha}$ and the relaxant effect of neomycin are shown in Figure 3.

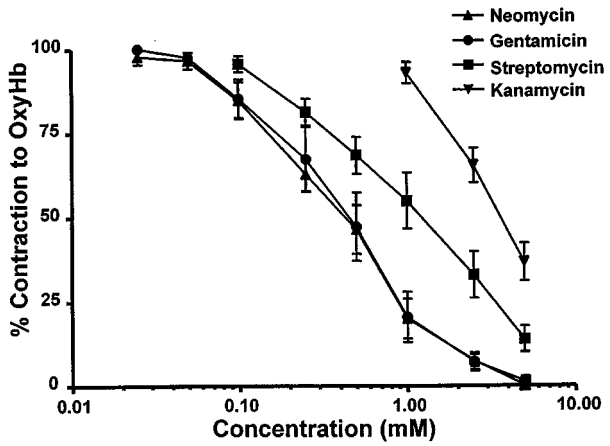

Figure 1 Cumulative concentration-effect curves for the relaxation of rings of canine basilar artery produced by the aminoglycosides. The preparations were precontracted with OxyHb (10 μM). Ordinate: contraction expressed as % of the maximum tension induced by 10 μM OxyHb. Abscissa: concentration of the aminoglycosides (mM) on a logarithmic scale. Values represent the mean ± standard error of the mean for experiments conducted with five or more tissue preparations from four or more animals.

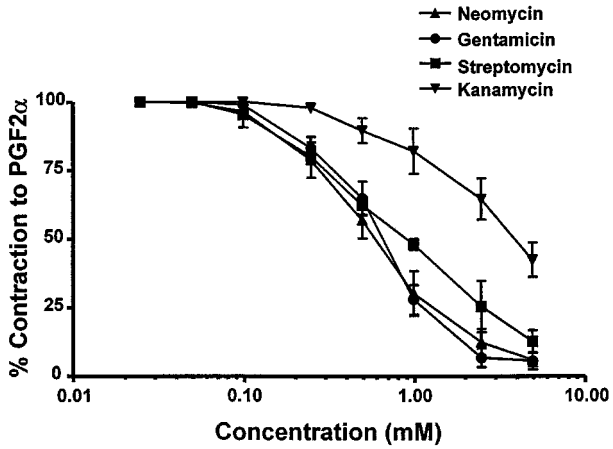

Figure 2 Cumulative concentration-effect curves for the relaxation of rings of canine basilar artery produced by the aminoglycosides. The preparations were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1 μM). Ordinate: contraction expressed as % of the maximum tension induced by 1 μM $PGF_{2\alpha}$. Abscissa: concentration of the aminoglycosides (mM) on a logarithmic scale. Values represent the mean ± standard error of the mean for experiments conducted with five or more tissue preparations from three or more separate animals.

Table 1 EC_{50} values for the aminoglycoside antibiotics against the vasoconstriction produced by the spasmogens oxyhaemoglobin, prostaglandin $F_{2\alpha}$ and potassium chloride

	Neomycin	*Gentamicin*	*Streptomycin*	*Kanamycin*
Oxyhaemoglobin	0.46 ± 0.10 ($n=6$)	0.53 ± 0.08 ($n=12$)	1.60 ± 0.29 ($n=7$)	3.88 ± 0.46 ($n=5$)
Prostaglandin $F_{2\alpha}$	0.89 ± 0.21 ($n=7$)	0.67 ± 0.07 ($n=5$)	0.90 ± 0.09 ($n=3$)	3.72 ± 0.64 ($n=4$)
Potassium Chloride	0.86 ± 0.14 ($n=5$)	1.12 ± 0.14 ($n=4$)	2.34 ± 0.75 ($n=5$)	4.51 ± 0.48 ($n=4$)

All the aminoglycosides examined produced relaxation of the contractions caused by 60 mM concentrations of potassium chloride, suggesting that in addition to their inhibitory action on PLC activity, these agents also inhibit calcium influx mediated *via* voltage-dependent calcium channels activated by depolarizing concentrations of KCl. The maximal relaxant effects produced by neomycin, gentamicin, streptomycin and kanamycin on KCl-mediated vasoconstrictions were $95.6 \pm 1.3\%$ ($n=5$), $96.8 \pm 3.2\%$ ($n=4$), $69.8\% \pm 6.9$ ($n=5$) and 52.8% ($n=5$), respectively (Figure 4). The EC_{50}s for these agents are shown in Table 1.

Time course of the vasorelaxant effects of the aminoglycosides

The persistence of the vasodilation produced by the aminoglycosides is potentially important, since experience with reversal of vasospasm in humans has shown that some agents such as papaverine can relax spastic vessels, but the response is too transient to be clinically useful (Kassell *et al.*, 1992). Since kanamycin has been shown to be effective in the reversal of angiographic spasm in the primate model of vasospasm (Zervas *et al.*, 1974) this aminoglycoside was used in the present study. Figure 5 shows representative traces of the effects of kanamycin on canine basilar artery, recorded over a time period of at least 3 h. Similar observations were made with regard to the time course of the other aminoglycosides. Relaxation of the sustained contraction to OxyHb is maintained over the entire period of time of the experiments. After 3 h, the magnitude of the relaxation was unchanged, an observation which may have important clinical implications.

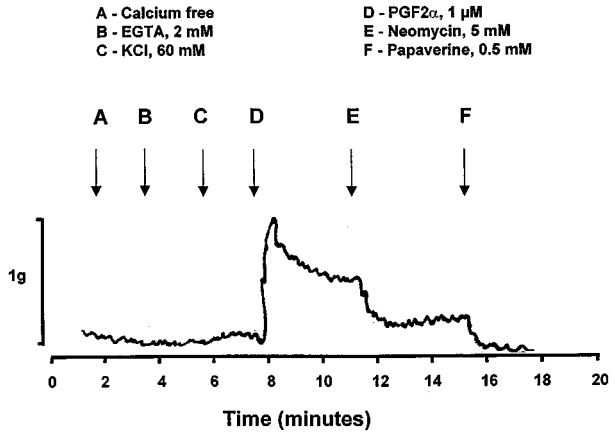

Figure 3 Representative traces of the response of ring preparations of canine basilar artery precontracted with prostaglandin $F_{2\alpha}$ (1 μM) in calcium-free medium. Each tracing illustrating the response of an individual tissue is representative of three independently conducted experiments with tissues from three separate animals.

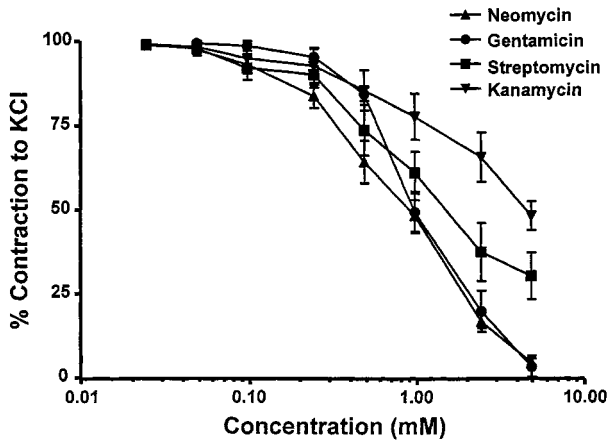

Figure 4 Cumulative concentration-effect curves for the relaxation of rings of canine basilar artery produced by the aminoglycosides. The preparations were precontracted by potassium chloride (KCl) (60 mM). Ordinate: contraction expressed as % of the maximum tension induced by 60 mM KCl. Abscissa: concentration of the aminoglycosides (mM) on a logarithmic scale. Values represent the mean ± standard error of the mean for experiments conducted with five or more tissue preparations from three or more separate animals.

Effect of vascular endothelium on vasodilation caused by the aminoglycosides

To determine whether the effectiveness of the aminoglycosides could arise from an effect mediated by the endothelium, we determined the effects of neomycin on the OxyHb-induced vascular tension in the isolated basilar artery, in the presence or absence of endothelium. Responses to bradykinin were recorded as an indicator of the preservation of the endothelium; in preparations with an intact endothelium bradykinin is vasorelaxant, while in preparations denuded of endothelium, the compound is a vasoconstrictor (Tsuji *et al.*, 1990). In Figure 6, representative responses to bradykinin are shown, recorded from canine arterial rings preconstricted with OxyHb (10 μM) (Figure 6A) or $PGF_{2\alpha}$ (1 μM) (Figure 6B). In the preparations in which endothelium had been removed, bradykinin had no relaxant effect, but produced a contraction at higher concentrations, while neomycin retained its full activity.

The effects of the aminoglycosides on intracellular calcium levels

Our previous studies have shown that in cerebrovascular smooth muscle cells OxyHb produces an increase in $[Ca^{2+}]_i$ levels 30 s after administration, which is maintained at 3 and

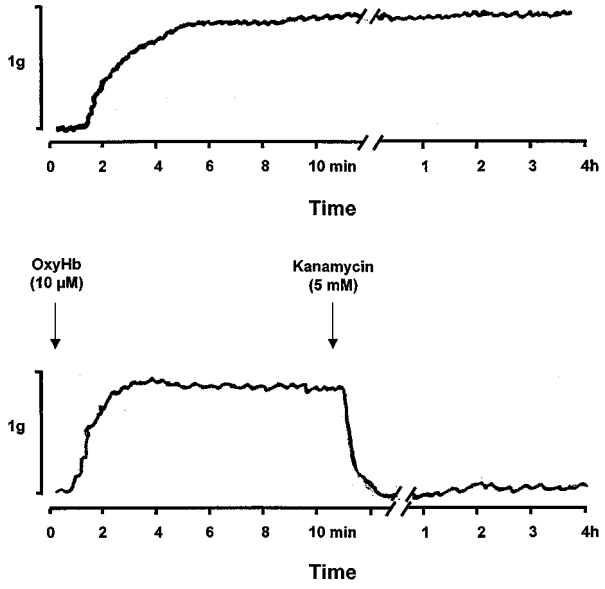

Figure 5 Representative traces of the effects of OxyHb (10 μM) alone (upper trace), and the effects of kanamycin (5 mM) on the responses of ring preparations pretreated with OxyHb (10 μM), followed over a time period of 3 h (lower trace).

24 h. This elevation was reduced after pretreatment with neomycin (Vollrath *et al*., 1994). In the present studies we have attempted to characterize the effects of neomycin and related aminoglycoside antibiotics on $[Ca^{2+}]_i$ levels by using cultured cerebrovascular smooth muscle cells pretreated with OxyHb.

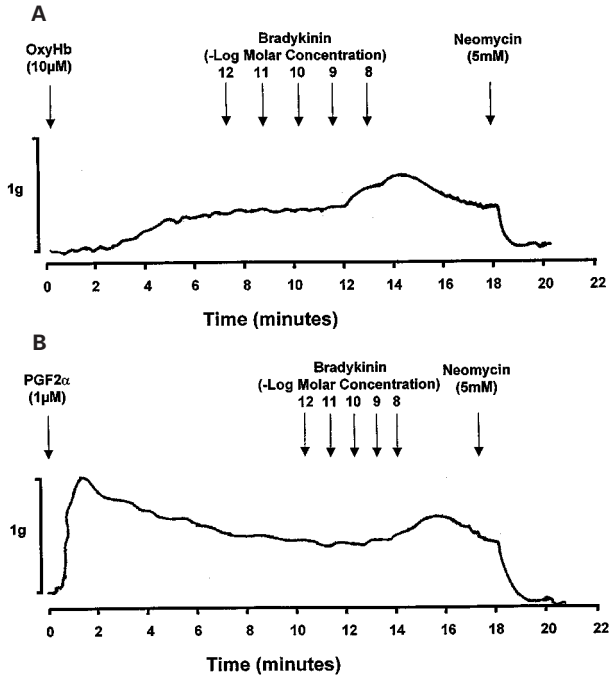

Figure 6 Representative traces of the effects of bradykinin on rings of canine basilar artery from which the endothelium had been mechanically removed. The rings were precontracted with OxyHb (10 μM) (upper trace) or prostaglandin $F_{2\alpha}$ (1 μM) (lower trace). Bradykinin produces no relaxation, and at higher doses produces a contraction, suggesting that the endothelium has been removed from the preparations. The relaxation of neomycin is unimpaired. Each trace, which illustrates the response of an individual ring preparation is representative of three independent experiments conducted with preparations from three different animals.

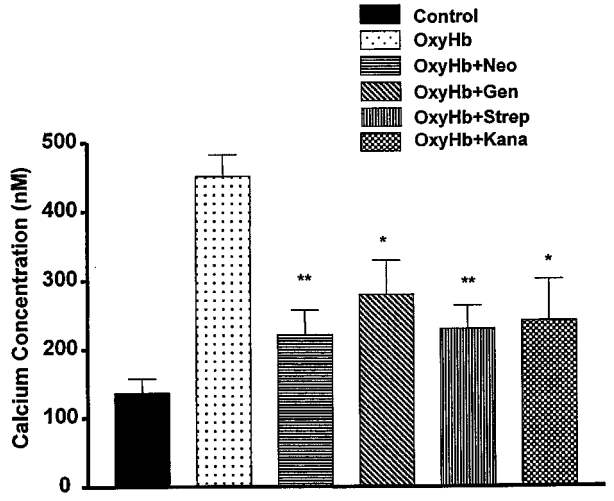

Figure 7 Effects of the aminoglycosides on the elevation of intracellular calcium produced in canine cerebrovascular smooth muscle cells by exposure to OxyHb (1 μM) for 24 h. The aminoglycosides, neomycin (Neo), gentamicin (Gen), streptomycin (Strep) and kanamycin (Kana) were administered at a concentration corresponding to their EC_{50} measured in contractility experiments, 3 h after the OxyHb application. Data presented are the mean $\pm$ standard error of the mean. $^*P<0.05$, $^{**}P<0.01$ compared to the response to OxyHb.

The cells were exposed to OxyHb (1 μM) for 24 h and the aminoglycosides, neomycin, gentamicin, kanamycin and streptomycin were administered 3 h. later in concentrations corresponding to the EC_{50}s determined in the contractility studies. As shown in Figure 7, OxyHb caused a highly significant increase in the level of intracellular calcium from control levels of 135.8 ± 21 nM, to 450 ± 31.8 nM ($n=8$). Administration of neomycin, gentamicin, kanamycin or streptomycin reduced these increased levels of calcium by about 50% to 221 ± 35.6 nM ($n=7$), 279.2 ± 31.3 nM ($n=7$), 229.1 ± 33.8 nM ($n=6$) and 240 ± 59.8 nM ($n=5$), respectively, an observation which corresponds to the results obtained from experiments in which contractility was measured.

Discussion

In these studies we have shown that OxyHb, a factor implicated in the pathogenesis of cerebral vasospasm, induced a sustained vasoconstriction of preparations of cerebral artery, which was reversed by the aminoglycoside antibiotics neomycin, gentamicin, kanamycin and streptomycin. Our studies also revealed that the aminoglycoside antibiotics were effective in relaxing cerebral arteries preparations preconstricted with $PGF_{2\alpha}$ and depolarizing concentrations of potassium chloride. The most potent vasorelaxants were neomycin and gentamicin while kanamycin and streptomycin were less effective. These compounds differ in the chemical nature and the number of positive charges, having six, five, four and two amino groups, respectively (Sande & Mandell, 1985). Thus, the inhibitory effects of the individual aminoglycosides seems to be, at least to some extent, a function of the number of amino groups. This observation is consistent with the results reported here concerning the inhibitory action of the aminoglycosides on the prolonged elevation of intracellular calcium in cultured cerebrovascular smooth muscle cells.

The chemical structure of the aminoglycosides suggests that they are unlikely to cross the cell membrane, so it is probable that they owe their effects to interation with anionic components of the cell surface. An immediate candidate for the mechanism by which the aminoglycosides cause relaxation of blood vessels is their known ability to inhibit PLC-mediated hydrolysis of inositol phospholipids and thus to prevent the subsequent elevation of $[Ca^{2+}]_i$ which is involved in smooth muscle contraction. It is well accepted that a rise in $[Ca^{2+}]$, which results from calcium mobilization from the sarcoplasmic reticulum in smooth muscle, leads to the binding of calcium to calmodulin and then to activation of myosin light-chain kinase (MLCK). Phosphorylation of myosin light chains by MLCK initiates myosin-actin interaction and activation of myosin ATP-ase activity which results in muscle contraction (Somlyo & Somlyo, 1994). Thus inhibition of PLC by the aminoglycosides would prevent the $Ins(1,4,5)P_3$-induced mobilization of $[Ca^{2+}]_i$ and thus inhibit the contraction. OxyHb has been shown to increase $Ins(1,4,5)P_3$ as well as $[Ca^{2+}]_i$ in cerebrovascular smooth muscle cells by a mechanism which is still unclear but which probably involves free radical-stimulated activation of PLC (Vollrath *et al*., 1994; Cook & Vollrath, 1995). We have shown previously that elevation of $Ins(1,4,5)P_3$ induced by OxyHb, may be prevented by neomycin (Vollrath *et al*., 1990), an observation which suggests that the contractile action of OxyHb may be mediated, at least in part, by PLC (Vollrath *et al*., 1994). Our observation that the aminoglycosides inhibit the action of prostaglandin $F_{2\alpha}$ in the absence of external calcium, supports the concept that the aminoglycosides owe part of their action to inhibition of PLC.

The effects of $PGF_{2\alpha}$ are mediated by receptors coupled to a Gq protein (Smith, 1989), and hence to an elevation of $Ins(1,4,5)P_3$ mediated by PLC_β the process also involves a PLC-mediated increase in $Ins(1,4,5)P_3$ and an elevation of $[Ca^{2+}]_i$ as described above. This process is independent of the presence of extracellular calcium (Berridge, 1993). Later in the development of the response, this initial elevation of $[Ca^{2+}]_i$ may activate a receptor or second messenger-operated Ca channel (Fasolato *et al*., 1994), but the initial response is maintained in a Calcium-free medium, and that response is inhibited by the aminoglycosides.

The mechanism involving an increase in levels of $Ins(1,4,5)P_3$ can account for only part of the action of the aminoglycosides; the elevation of $Ins(1,4,5)P_3$ is transient and it is unlikely that the sustained response to OxyHb, which corresponds to a large and sustained increase in $[Ca^{2+}]_i$, arises from activation of PLC alone. The studies reported here show that the sustained elevation of $[Ca^{2+}]_i$ produced by OxyHb can be reversed by the aminoglycosides, when they are administered after the concentration of $Ins(1,4,5)P_3$ has returned to the control level. This clearly suggests that other sites of action should be considered, a view which is further supported by the observation that all aminoglycosides used in these studies inhibited vasoconstriction to depolarizing concentrations of potassium chloride, which cause contraction by increasing calcium influx through L-type voltage-dependent calcium channels, a process which is independent of the activation of PLC. Since molecular events leading to uptake and binding of Ca^{2+} are thought to involve association of Ca^{2+} with anionic binding sites localized in calcium channel subunits (Catterall & Striessnig, 1992), it is conceivable that the availability of channel anionic sites may be decreased by polycationic organic bases such as the aminoglycosides, thus resulting in the inhibition of calcium entry (Catterall & Striessnig, 1992). On the other hand, calcium influx through channels of this type does not seem to play a major role in cerebral vasospasm or in OxyHb-mediated vasoconstriction, since antagonists of L-type channels are rather poor antagonists of both processes (Espinosa *et al*., 1984; Krueger *et al*., 1985). Nimodipine was originally recommended for the clinical management of vasospasm because it was believed that it would relax the constricted blood vessels; it now appears most likely that the benefit of nimodipine in severe vasospasm arises from a neuroprotective effect on ischaemic regions thus limiting the size of the infarct (Robinson & Teasdale, 1990). Significant vasodilation during clinical vasospasm has been difficult to demonstrate, and although the issue remains controversial, nimodipine in our hands is not very effective at reversing the OxyHb-induced contraction of isolated arterial segments (Krueger *et al*., 1985; Vollrath *et al*., 1990).

Neomycin and the other aminoglycosides are much more effective in reversing OxyHb-induced contractions than the L-type calcium channel antagonists, and there are a variety of possible explanations for this. First, neomycin is a known inhibitor of L-type calcium channels (Keith *et al*., 1992, Langton *et al*., 1996), but has also been reported to inhibit a variety of ion channels in addition to the L-type including N-, and 'non-L/non-N'-type voltage sensitive calcium channels (Keith *et al*., 1992; Pichler *et al*., 1996). The exact nature of the mechanism by which cells treated with OxyHb accumulate calcium is not known, however, and electrophysiological studies in freshly dissociated cells revealed a calcium influx in response to OxyHb which had few characteristics of known calcium transport mechanisms (Steele *et al*., 1991). It is also possible that the lesion lies not in an acceleration of calcium influx, but an inhibition of calcium efflux, a suggestion for which there is some experimental evidence (Wang *et al*., 1994). The plasma membrane Ca^{2+} pump can be damaged by the action of free radicals (Rohn *et al*., 1996), and this protein is the preferred substrate for the proteolytic action of calpain, a protease activated by high levels of $[Ca^{2+}]_i$ (Salomino *et al*., 1994). It is conceivable that aminoglycosides have some protective effects on this process, possibly by forming a ternary complex with iron and the phospholipid polar head, which may decrease Fe^{2+} oxidation and thus inhibit the production of oxygen free radicals, as has been observed for endogenous polyamines (Tadolini, 1988).

Another possibility for the action of the aminoglycosides involves an endothelium-mediated relaxation. The haeme moiety of OxyHb molecule is known to bind nitric oxide, a diffusable vasodilator synthesized from arginine in endothelial cells; OxyHb would thus prevent the relaxant effect of nitric oxide in vascular smooth muscle (Moncada *et al*., 1991). It has been suggested that this mechanism might, in part, explain the vasoconstriction observed with OxyHb (Vane *et al*., 1990). In the present studies, we have shown that removal of the endothelium prevented bradykinin-mediated vasodilation, but did not affect the vasorelaxant action of the aminoglycosides. This explanation thus seems unlikely.

It is possible that the unexpected effectiveness of the aminoglycosides in preventing the action of OxyHb, arises from a multiplicity of actions. If they inhibit the action of $PGF_{2\alpha}$ by acting as inhibitors of phospholipase C, and they block potassium by interfering with calcium influx, it is possible that both effects come into play in the case of OxyHb. There is reason to believe that the initial and the sustained contractions observed with OxyHb are not independent processes; it may be that the sustained calcium entry is triggered by the early elevation in $Ins(1,4,5)P_3$, and if this is true, the combination of two separate mechanisms may account for the high effectiveness of this group of compounds. Currently the only clinical data is for kanamycin, a compound which was shown to be effective in whole animal models and in patients about two decades ago. In our studies this was actually the least effective aminoglycoside, and thus studies in whole animal models of vasospasm with more potent aminoglycosides or other polycationic compounds are well worth undertaking. It is unlikely that i.v. administration of these drugs would provide access to the spastic vessels in sufficient concentration to provide effective relaxation, since the drugs would not be expected to cross the blood-brain barrier. On the other hand, intracranial application of a sustained release preparation during surgery for clipping of the aneurysm (Kasuya *et al*., 1997), remains a realistic possibility.

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