UNIVERSITY OF ALBERTA

CEREBROVASCULAR EFFECTS OF POTASSIUM CHANNEL MODULATORS AND THEIR POSSIBLE ROLE AGAINST CEREBRAL VASOSPASM

BY

He Zhang

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The undersigned certify they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Cerebrovascular Effects of Potassium Channel Modulators and Their Possible Role Against Cerebral Vasospasm** submitted by **He Zhang** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Experimental Surgery (Electrophysiology and Pharmacology).**

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This work is dedicated to my wife, Jiping,
and my daughter Jennifer

ABSTRACT

Cerebral vasospasm is a prolonged contraction of the cerebral vessels which frequently occurs a few days after subarachnoid hemorrhage and for which no effective treatment is currently available. Agents which modulate potassium conductance are of potential use in this condition, and we have thus examined potassium channels and the action of drugs which modulate them in the cerebral vasculature.

Primary isolates of rat basilar artery smooth muscle cells were studied using whole-cell and single-channel patch-clamp techniques. Two distinct potassium currents were identified, a delayed rectifier potassium current sensitive to procaine or strychnine, a calcium-activated potassium current sensitive to tetraethylammonium (TEA) or charybdotoxin. A small inward current was activated by hyperpolarization beyond -80 mV.

The effects of the potassium channel agonists nicorandil, pinacidil and cromakalim were examined on the delayed rectifier and calcium-activated potassium currents. Only the calcium-activated potassium current was increased by high concentrations of cromakalim and pinacidil, while neither component was affected by nicorandil. In single channel recording, cromakalim and pinacidil increased the opening probability of the large-conductance calcium-activated potassium channel, while nicorandil had no consistent effects.

Nicorandil, pinacidil and lemakalim (the vasorelaxant (-)-trans enantiomer of cromakalim) relaxed precontracted rings of canine cerebral artery. The order of potency was lemakalim > nicorandil ≅ pinacidil. The effects of nicorandil were inhibited by methylene blue but not by glibenclamide, while the effects of pinacidil and lemakalim were inhibited by glibenclamide but not by methylene blue. Thus nicorandil probably causes relaxation mostly by effects on guanylate cyclase while lemakalim and pinacidil produce the same effect by action at potassium channels.

Iloprost, which was suggested to produce relaxation by opening potassium channels, was studied to determine its potential use against vasospasm. Iloprost caused

relaxation of canine cerebral arteries precontracted with oxyhemoglobin, prostaglandin $F_{2\alpha}$ or the thromboxane A_2 analogue U 46619, but not with KCl. The presence of a moderate delayed vasospasm in the tested arteries provided similar results to those obtained in control arteries. It is possible that iloprost may play a role in the treatment of vasospasm after subarachnoid hemorrhage.

Glibenclamide, previously described as a specific antagonist of ATP-dependent potassium channels, unexpectly, was found to relax contractions induced by prostaglandin $F_{2\alpha}$ in rat aorta, and in canine femoral, mesenteric, renal, coronary, basilar and middle cerebral arteries. The action of glibenclamide on cerebral arteries was not mediated by cGMP as it was not blocked by methylene blue, an inhibitor of guanylate cyclase, nor was the relaxation endothelium-dependent. Glibenclamide relaxed contractions of canine middle cerebral artery induced by prostaglandins $F_{2\alpha}$, E_2 and D_2, or U46619, but not by KCl, noradrenaline, 5-hydroxytryptamine or caffeine. In vascular preparations contracted with oxyhemoglobin, glibenclamide also causes relaxation, and since the only other agents sensitive to glibenclamide are eicosanoids, this observation supports the idea that there is some link between responses to oxyhemoglobin and the production of eicosanoids. Several other sulphonylureas were studied for their effects on eicosanoids using rat aorta. The results suggested that tolbutamide, glipizide and glimepiride have actions similar to glibenclamide, while chlorpropamide lacks antagonist activity.

These agents which modulate potassium channels have important and sometimes unexpected activity in cerebral vessels, and may have some potential value in the management of cerebral vasospasm.

KEY WORDS

Potassium Channel; Potassium Channel Agonists; Iloprost; Sulphonylureas, Eicosanoids; Oxyhemoglobin; Vasospasm

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CHAPTER ONE

PATHOGENESIS OF CEREBRAL VASOSPASM

Following the rupture of an aneurysm in the cerebral circulation a delayed and long lasting contraction of the arteries surrounded by the perivascular blood clot can occur. This is known as cerebral vasospasm. The first case of cerebral vasospasm was described in a young woman by an English physician Gull (1859). *On the fifth day following the onset of her illness, she could utter a few words. The following day, however, her condition again deteriorated, and she died. At autopsy, a ruptured middle cerebral artery aneurysm was found embedded in a large sylvan clot and the adjacent hemisphere showed massive softening.*

The history of cerebral vasospasm was later systematically reviewed by Weir (1990), who particularly summarized the clinical and research advances made in the last four decades. Two important publications on the pathogenesis of vasospasm in 1949, led to the hypothesis that the red blood cells were responsible for the greatest meningeal response, and the ischemic changes were due to temporary spasm of the vessels rather than compression of the arteries by the aneurysm. Subsequently, Ecker and Riemenschneider (1951) provided the first description of angiographic spasm of the intracranial arteries and established its relationship to rupture of an intracranial aneurysm. With the consensus that vasospasm was a major cause of morbidity and mortality following aneurysmal rupture in 1960s, our knowledge of the significance of angiographic vasospasm in production of delayed ischemic neurologic deficits rapidly accelerated during 1970s. The studies of etiology, the application of early operation, the uses of computer-assisted tomography and positron emission tomography have all contributed to our knowledge of vasospasm.

From the 1980s to the present, numerous reviews have been published concerning nearly every aspects of the study of cerebral vasospasm (Cook, 1984; Kassell et al., 1985; Wellum et al., 1985; Wilkins, 1986; Weir, 1987; Kiwak and Heros, 1987; Bevan and Bevan, 1988; Weir, 1990; Findlay et al., 1991; Macdonald and Weir, 1991). The

pathogenesis of cerebral vasospasm has been reviewed recently and it is clear that based on current experimental and clinical evidence, oxyhemoglobin is the principal pathogenetic agent responsible for cerebral vasospasm (Macdonald and Weir, 1991). The mechanism by which oxyhemoglobin produces vasospasm is unclear. Oxyhemoglobin releases free radicals, initiates and propagates lipid peroxidation, releases vasoactive eicosanoids and endothelin, elevates intracellular IP_3 and then increases intracellular calcium concentration, produces perivascular nerve damage and induces structural damage in the arterial wall.

This introduction is divided into two main sections: *a.* a brief summary of the pathogenesis of cerebral vasospasm; *b.* the potassium channel modulators and their possible usage against vasospasm. In this chapter and indeed, throughout the experimental studies performed which comprise the body of this thesis, I would like to present the hypothesis that the potassium channels may play a significant role in the regulating of cerebral arterial smooth muscle tone and those potassium channel modulators could be useful in the prevention and treatment of cerebral vasospasm.

GENERAL INFORMATION

Definitions. Sustained cerebral arterial narrowing occurring days after subarachnoid hemorrhage is commonly referred to as cerebral vasospasm. The cerebral spasm, sometimes called cerebrovascular spasm, usually occurs at a site where the arterial wall has been weakened and an aneurysm has formed, but does not involve the aneurysm itself because of the lack of innervation and muscle (Weir, 1987). Aneurysms are most often found at the bifurcations of the cerebral arterial tree, where shear stress from turbulent blood flow is at its greatest. The cause of rupture is most commonly associated with episodes of elevated blood pressure. Rupture commonly occurs at the internal carotid, anterior or middle cerebral arteries or in the communicating arteries of the circle of Willis (Weir, 1987).

There are two definitions of cerebral vasospasm, angiographic and clinical, which may not be the same thing. Angiographic vasospasm is a narrowing of the dye column in

the major cerebral arteries which is usually focal but may be diffuse. The narrowing is time-dependent, rarely pronounced before the 4th day following the initial hemorrhage and reaches a peak around the 7th day. Clinical vasospasm is the syndrome of the ischemic consequences of cerebral arterial narrowing and is characterized by the insidious onset of confusion and decreased level of consciousness followed by focal motor and speech impairments and is often heralded by worsening headache and increasing blood pressure (Kassell et al., 1985). This delayed onset arterial narrowing is a hallmark feature of this condition, and thus is often described as chronic cerebral vasospasm. This must be distinguished from "acute" or "early" vasospasm, a narrowing of the cerebral arteries observed within the first few hours after blood application seen in experimental models of vasospasm. Whether the acute vasospasm occurs in man is less well established (Weir et al., 1978).

Incidence. Vasospasm occurs in a biphasic fashion. The acute phase occurs within minutes of the subarachnoid hemorrhage and may serve as a protective mechanism by reducing blood flow to the aneurysm site and preventing rebleed. The chronic phase begins 2-24 hours after the initial bleed, becomes apparent after 3 days and may persist for weeks posing a serious threat to the patient's survival and neurological recovery (Cook, 1984). Aneurysmal subarachnoid hemorrhage accounts for approximately 6-8% of all strokes. Arteriographic vasospasm can be detected on about one-half of the arteriograms obtained from 4-12 days after subarachnoid hemorrhage. However, only 20-30% of patients will suffer a delayed neurological deterioration related to ischemia (Kiwak and Heros, 1987). Ruptured intracranial aneurysm is a major public health problem affecting more than 28,000 individuals in North America each year. In excess of 19, 000 will ultimately die or be disabled (Kassell et al., 1985). Currently, about half of the patients die within 3 months, half of the survivors have a major disability, and about two thirds of those who have successful aneurysm obliteration never regain their prehemorrhage quality of life (Drake, 1981).

Pathology. The pathological changes of human cerebral vessels that have been in spasm indicate that between several days and several weeks after subarachnoid hemorrhage, some smooth muscle cell necrosis occurs, leading to a reduction in the number of layers of smooth muscle cells in the media (Hughes and Schianchi, 1978). Swelling of the intima becomes apparent during this time and after several weeks develops into a sub-endothelial fibrosis that narrows the lumen (Conway and Mcdonald, 1972; Hughes and Schianchi, 1978). Findlay et al. (1991) reviewed the structure changes during cerebral vasospasm and discussed two hypotheses, prolonged vasoconstriction and structure change. They suggested that vasospasm represents sustained arterial contraction rather than structural thickening of the vessel wall with lumen encroachment, which includes intimal proliferation, inflammation and arterial wall fibrosis. Another hypothesis is that the chronic arterial narrowing is not maintained by continuous active vascular smooth muscle contraction, but by the structural change in the cerebral artery wall resulting from cell damage (Bevan and Bevan, 1988). These authors emphasize the important relation between the early reversible vasospasm and the late irreversible arterial narrowing, and the implications of structural change.

Prevention and Treatment. The prevention or treatment of cerebral vasospasm has been reviewed by Wilkins (1973, 1980, 1986). The current management strategies for vasospasm can be broadly divided into two parts: preventing or reversing arterial narrowing and management of the resultant cerebral ischemia (Findlay et al., 1991). Subarachnoid clot removal (Nosko et al., 1987), pharmacological intervention (Cook, 1984) with such agents as the nonglucocorticoid 21-aminosteroid U74006F, which is an inhibitor of iron-dependent lipid peroxidation (Kanamaru et al., 1990; Steinke et al., 1989; Zuccarello et al., 1989), the iron chelator deferoxamine (Vollmer et al., 1991), or the calcium channel antagonists (Allen and Bahr, 1979) and percutaneous transluminal angioplasty (Zubkov et al., 1984) provide the principal strategies for management of arterial narrowing. The treatment of cerebral ischemia includes hypertensive, hypervolemic

and hemodilutional therapy and also the application of calcium channel antagonists.

Experimental Models. One difficulty of the study of this disorder is the lack of an entirely suitable animal model which accurately reflects the situation in humans. The greatest difficulty has been experienced in reproducing the late-onset vasospasm where delayed neurological deficits occur and can be observed. Although the experiments have been carried out in monkey, baboon, dog, cat, rabbit, or rat (Bevan and Bevan, 1988), the animal model which most closely approximates the human condition is that using the cynomolgus monkey, in which an autologous blood clot is surgically packed around the middle cerebral artery, a method which was developed at this university (Espinosa et al., 1984; Nosko et al., 1985). This is reported to be the first unequivocal example of delayed ischemic deficits associated with vasospasm occurring in an animal model of vasospasm (Weir, 1987).

A variety of *in vitro* approaches have been used which include pharmacological studies of blood vessel contractility using vessel segments or perfused arterial preparations, biochemical investigations of the artery and isolated smooth muscle cells from cerebral arteries. It has been suggested that the shortcoming of all the *in vitro* studies is that none of the methodologies specifically address the delayed cerebrovascular spasm and thus may only provide us with knowledge of acute vasospasm. The validity of this objection depends on the reason for the delay. If the delay in spasm aries from the slow release of a mediator which acts immediately, then isolated preparations may provide a useful model. If the delay arises because, for some reason, the spasmogen takes several days to produce a response, the *in vitro* approaches would be less significant. There is evidence that the slow release of oxyhemoglobin from lysed erythrocytes in the subarachnoid clot is a mayor determinant in the delay (*vide infra*). In addition, there is a suggestion that the delayed constriction of cerebral arteries in spasm is not an active contraction but a passive, irreversible state as a result of processes which begin during the early stages following subarachnoid hemorrhage (Bevan and Bevan, 1988). For these reasons the usefulness of the *in vitro* study outweighs

the disadvantages. Thus, the pharmacological studies of blood vessel contractility and patch-clamp study of potassium channels in isolated smooth muscle cells have been employed in this thesis.

PATHOGENESIS OF CEREBRAL VASOSPASM

Potential Spasmogens. There are only three logical sites to look for a spasmogen: the blood (fresh and/or clotted), the cerebrospinal fluid, and the arterial wall (Cook, 1984). The etiologic role of subarachnoid blood clot in producing arterial constriction after subarachnoid hemorrhage suggests that a compound is released from clot that interacts in some way with the vessel wall, producing vasoconstriction which becomes irreversible, with delayed onset and prolonged duration. The time course could be explained by a spasmogen exhibiting a similar time course of appearance in cerebrospinal fluid adjacent to arteries in spasm (Findlay et al., 1991). Erythrocyte hemolysis in cerebrospinal fluid is the most prominent process during vasospasm, which release of oxyhemoglobin and its breakdown products, bilirubin and methemoglobin (Barrows et al., 1955; Duff et al., 1988). In addition to these substances, other vasoactive agents such as lipid peroxides (Sano, 1988), eicosanoids (Chehrazi et al., 1989) and endothelin (Masaoka et al., 1990) are present in cerebrospinal fluid following subarachnoid hemorrhage.

Hemoglobin. Hemoglobin is believed to be the cause of vasospasm (Macdonald and Weir, 1991). Hemoglobin, and particularly oxyhemoglobin, contracts isolated cerebral arteries of several different animal species. Fujii and Fujitsu (1988) reported that oxyhemoglobin contracted cultured smooth muscle cells of rat aorta. Vacuolation, cell degeneration, and loss of internal cell structure were noted after 24 h of exposure. Electrophysiological examination revealed that oxyhemoglobin contracted smooth muscle cells isolated from rat basilar artery and increased calcium-activated potassium currents (Steele et al., 1990). Oxyhemoglobin increased the release of inositol trisphosphate (IP_3) from vascular smooth muscle which in turn releases calcium from intracellular stores (Vollrath et al., 1990).

In isolated arteries, Cook et al. (1979) demonstrated that hemoglobin causes slowly developing and long-lasting contraction of dog cerebral arteries, rabbit ear arteries, and rat stomach fundus. Similar results were obtained from other studies (Macdonald and Weir, 1991). A piglet model of subarachnoid hemorrhage has been developed in which high concentration of hemoglobin or various blood components were maintained adjacent to the right middle cerebral artery for 10 days (Mayberg et al., 1990a,b). A significant decrease in lumen area was observed. Oxyhemoglobin and supernatant fluid, but not pure methemoglobin, produced significant vasospasm in cynomolgus monkeys model of subarachnoid hemorrhage (Macdonald et al., 1991).

The mechanism by which hemoglobin produces vasoconstriction remains unclear. Contraction caused by receptor-operated systems usually decreases with time due to tachyphylaxis, desensitization, and/or autoregulation (Cook, 1984; Wellum et al., 1985). The time course of vasospasm suggests that smooth muscle contraction by a conventional receptor-operated system is not the sole mechanism responsible for arterial narrowing (Macdonald and Weir, 1991). The agents that acting on cell surface receptors, such as serotonin, other biogenic amines, peptides, and so on, are unlikely to be the sole mediators of vasospasm. The negligible therapeutic benefit in vasospasm, achieved by treatment with antagonists of receptor-operated vasoconstrictors, including such drugs as atropine, methysergide, cinanserin, ketanserin, phenoxybenzamine, phentolamine, mepyramine, chlorpheniramine, propranolol, salbutamol, sarcosine, alanine, theophylline, and quinine, supports this idea (Macdonald and Weir, 1991). Even the results obtained with papaverine and the calcium channel antagonists are variable.

Eicosanoids. The 20-carbon fatty acid arachidonic acid is the most common fatty acid found in the phospholipids of mammalian cells. All products of its metabolism are collectively known as the eicosanoids. These include the prostaglandins, thromboxanes, leukotrienes, lipoxins, hydroperoxy- and hydroxy-eicosatetraenoic acids (Walker and Pickard, 1985). Cerebral arteries contract in response to most prostaglandins except for

prostacyclin, which causes vasodilation (Cook, 1984). Cyclooxygenase metabolites of arachidonic acid have long been implicated in the development of cerebral vasospasm.

The hypothesis that cerebral vasospasm could arise from an imbalance between prostacyclin and thromboxane synthesis has received considerable attention. Decreased vasodilator influence from prostacyclin, combined with endothelial damage with adherence of platelets and release of vasoconstricting prostaglandins and thromboxane, could contribute to vasospasm. After subarachnoid hemorrhage in monkeys, vasospastic vessels synthesize less prostacyclin and more vasoconstricting prostaglandins (Nosko et al., 1988; Chehrazi, 1989; White, 1990). There were no significant differences in thromboxane formation between the arteries from the experimental groups (Nosko et al., 1988). Patients with subarachnoid hemorrhage showing vasospasm had elevated cerebrospinal fluid levels of prostaglandins D_2 but not prostacyclin over patients with subarachnoid hemorrhage but no vasospasm (Rodriguez y Baena et al., 1987). These studies show that there is increased arachidonic acid metabolism in the subarachnoid space after hemorrhage, with prostaglandins being released and accumulating in the cerebrospinal fluid.

Canine cerebral arteries contract weakly in response to the peptidoleukotrienes (Jancar et al., 1987). Stimulation with arachidonic acid resulted in a time- and dose-dependent increase in the formation of both 5- and 15-hydroxy eicosatetraenoic acids (HETE), with the 15-hydroxy acid being most abundant. Thus, the contractile activity of arachidonic acid in cerebral arteries arises, at least in part, from hydroperoxyeicosatetraenoic acid (HPETE) formation (Schulz et al., 1989). A intermediary in the lipoxygenase pathway, 15-hydroperoxyeicosatetraenoic acid, is a potent vasoconstrictor and was generated in significant amounts by dog basilar artery during vasospasm (Cook and Schulz, 1990). Cerebrospinal fluid levels of 5-HETE or leukotriene C_4 were increased in patients with subarachnoid hemorrhage (Sasaki et al., 1982; Paoletti et al., 1988). It is possible that the products of the lipoxygenase arm of the

arachidonic acid cascade could play a role in the development of cerebral vasospasm (Cook and Schulz, 1990). The HPETEs may be generated initially by free radical reactions which occur during the breakdown of the blood clot and degradation of oxyhemoglobin to methemoglobin, with the red blood cell membrane acting as a source of arachidonate (Baker and Loh, 1987).

It is unlikely, however, that the presence of arachidonic metabolites in the cerebrospinal fluid accounts for all the facets of vasospasm. Inhibitors of prostaglandins and thromboxane synthesis have little effect on experimental and human vasospasm (Cook and Schulz, 1990; White, 1990). Since the effect of oxyhemoglobin on the vessel wall is possibly a combined actions of eicosanoids with free radicals, lipid peroxides and endothelin, antagonism of only one of these systems might not completely reverse the contraction.

Free Radicals. Oxyhemoglobin spontaneously autoxidizes to methemoglobin, releasing the superoxide anion radical (Misra and Fridovich, 1972; Wever et al., 1973). The superoxide anion radical conjuncted with iron could initiate and propagate lipid peroxidation by the Haber-Weiss reaction and Fenton chemistry (Sasaki et al., 1979; Asano et al., 1980). Lipid peroxides were suggested to cause vasoconstriction and structural damage to cerebral arteries both in vitro (Koide et al., 1982) and in vivo (Sasaki et al., 1980). Concentrations of lipid peroxides in the CSF of patients with SAH correlate with vasospasm (Misra and Fridovich, 1972; Sasaki et al., 1979; Sasaki et al., 1980). Furthermore, an inhibitor of iron-dependent lipid peroxidation, U74006F significantly diminished vasospasm in a primate model of SAH (Steinke et al., 1989).

Effects of the free radical-scavenging enzymes on the vasoactivity of oxyhemoglobin on cerebral arteries in vitro have been reported, but the results are inconsistent (Macdonald and Weir, 1991). Electrophysiological studies of isolated rat cerebral artery smooth muscle cells have shown oxyhemoglobin to activate calcium-dependent potassium currents and cause cell death (Steele et al., 1990). Catalase protected

cells from oxyhemoglobin, whereas superoxide dismutase did not. Xanthine and xanthine oxidase did not cause electrophysiological changes in cells, whereas the generation of hydroxyl radical in the bathing solution was damaging. The findings implicate the hydroxyl free radical rather than other oxygen free radicals although it may be difficult to define the actual free radicals involved based on such scavenger experiments (Halliwell and Gutteridge, 1986).

Bilirubin. Duff et al. (1988) reported that application of bilirubin solutions to exposed basilar arteries of cats and baboons resulted in progressive vasoconstriction that was associated with ultrastructural damage to the arterial wall. Electronmicroscopy of the arteries showed swelling of endothelial cells, degeneration of axons and varicosities in the adventitia, and extensive vacuolation of smooth muscle and endothelial cells. These results were supported by investigations by Mial and Lee (1989) that demonstrated the vasocontractile effects of bilirubin on cerebral arteries. Multiple intrathecal injections of bilirubin, however, failed to produce significant arterial narrowing in a monkey model, but did generate some pathological changes (Macdonald et al., 1991).

There are several reasons to question whether bilirubin could be an important spasmogen, although the time course of the appearance of bilirubin in the CSF after subarachnoid hemorrhage is similar to that of vasospasm (Macdonald and Weir, 1991). Bilirubin is found in the CSF in obstructive jaundice, yet vasospasm or focal neurologic deficits that might be related to vasospasm are rare in this condition. Production of bilirubin in the subarachnoid space is by an enzyme with limited capacity and may result in bilirubin concentrations that are too low to induce significant arterial narrowing after subarachnoid hemorrhage. The observation that intrathecal injections of oxyhemoglobin cause vasospasm whereas injections of methemoglobin do not, is also inconsistent with the bilirubin theory since both types of hemoglobin produce bilirubin in the subarachnoid space (Findlay et al., 1991).

Endothelium Factors. Endothelium factors include endothelium-dependent

relaxing factor (EDRF), endothelium-dependent hyperpolarizing factor (EDHF), prostacyclin and endothelin (Taylor and Weston, 1988; Nishiye et al., 1989; Moncada et al., 1991). Endothelium-dependent relaxation is inhibited after subarachnoid hemorrhage (Kim et al., 1988). Oxyhemoglobin but not methemoglobin is a potent inhibitor of endothelium-dependent relaxation and it is most likely to be the substance responsible for inhibition of endothelium-dependent relaxation after subarachnoid hemorrhage (Tsuji et al., 1989). EDRF may be nitric oxide (Moncada et al., 1991), and hemoglobin binds nitric oxide with an affinity 1500 times higher than its affinity for oxygen (Gibson and Roughton, 1957). Thus hemoglobin, which presumably does not enter cells, could prevent EDRF from entering smooth muscle cells and producing its effects. Hemoglobin prevents relaxation but does not alter hyperpolarization, which is produced by EDHF (Nishiye et al., 1989).

In addition to EDRF and EDHF, endothelial cells synthesize another vasodilator, prostacyclin. Synthesis of prostacyclin is diminished in vasospastic arteries and could contribute to vasospasm by disrupting the normal balance of constrictor and dilator functions within the arterial wall (Nosko et al., 1988). The endothelium-dependent constricting factor, endothelin has also been associated with vasospasm (Kobayashi et al., 1991). Although alteration of endothelial function could contribute to vasospasm, evidence suggests these effects are of secondary importance compared with effects of spasmogens directly on smooth muscle (Findlay et al., 1991).

Neurogenic Effects. Neurogenic mechanisms in the pathogenesis of vasospasm has been reviewed previously (Wellum et al., 1985). Although cerebral arteries receive adrenergic, cholinergic, and peptidergic innervation and possess receptors for neurotransmitters such as serotonin, α- and β-adrenergic drugs, dopamine, and histamine, the precise role of these nerves and receptors in the regulation of cerebrovascular tone is unknown (Macdonald and Weir, 1991). Conflicting results have been reported about the effects of subarachnoid hemorrhage on these nerves, and neither cervical sympathectomy nor pharmacologic intervention are effective. Despite contradictory evidence regarding the

significance of changes in cerebrovascular innervation in relation to vasospasm, subarachnoid hemorrhage clearly does damage these nerves and oxyhemoglobin may be responsible for this degeneration (Linnik and Lee, 1989).

Thus although oxyhemoglobin is probably the agent responsible for delayed arterial spasm, neither its mechanism of action nor ways to antagonize or abort its actions are well understood.

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CHAPTER TWO
VASCULAR EFFECTS OF POTASSIUM CHANNEL MODULATORS AND THEIR POSSIBLE ROLE AGAINST CEREBRAL VASOSPASM

The membrane potential of cerebral arterial smooth muscle is depolarized during the vasospasm (Varsos et al., 1983) which follows about 50% of the cases of subarachnoid hemorrhage (Kiwak and Heros, 1987). This depolarization may play a role in prolonged constriction and vascular hyperreactivity which underlies cerebral vasospasm (Varsos et al., 1983; Waters and Harder, 1985; Peterson et al., 1985). The potassium channel agonist, nicorandil, hyperpolarizes the smooth muscle membrane by increasing potassium conductance (Furukawa et al., 1981) and, at least partly, reverses the vasospasm in a canine model of subarachnoid hemorrhage (Harder et al., 1987). The mechanisms of opening potassium channels which initiate the relaxation of vascular smooth muscle have been discussed by many authors (Cook, 1988a; Weston, 1988; Hamilton and Weston, 1989; Edwards and Weston, 1990a; Cook and Quast, 1990; Kajioka et al., 1991). When a potassium channel opens, potassium diffuses down its electrochemical gradient, transferring positive charge out of the cell, thereby making the interior of the cell more negative and driving the membrane potential in an hyperpolarizing direction (Blatz and Magleby, 1987). Potassium channel agonists initiate other events, such as inhibition of the refilling of calcium stores (Bray et al., 1988a) and perhaps increasing of calcium extrusion via voltage-dependent sodium-calcium exchange (Kaczorowski et al., 1988). All these events will result in the reduction of intracellular calcium concentration and muscle relaxation.

In this brief review, we summarize the studies of the properties of potassium

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channels in vascular smooth muscle, especially those in cerebrovascular smooth muscle. We also address the mechanisms and action of potassium channel modulators. We focus attention on the effects of potassium channel modulators in cerebrovascular smooth muscle and their possible usage in the treatment of vasospasm which follows subarachnoid hemorrhage.

VASCULAR SMOOTH MUSCLE POTASSIUM CHANNELS

Potassium channels, unlike most other ionic channels, are present in nearly all cell types where they play very different physiological functions. They are usually classified by their mode of activation. Some are activated strictly by variations in membrane potential, others by variations of intracellular concentrations of calcium, some by internal concentrations of ATP, sodium or cyclic nucleotides (Rudy, 1988; Cook, 1988a; Castle et al., 1989; Edwards and Weston, 1990a). Based on their gating, conductance and pharmacological characteristics, potassium channels in vascular smooth muscle can be divided into four basic groups; voltage dependent, calcium sensitive, receptor coupled and "other" potassium channels. This classification relates only to those different potassium channels characterized in patch-clamp studies of vascular smooth muscle.

VOLTAGE-DEPENDENT POTASSIUM CHANNELS

Voltage dependent potassium channels can be further subdivided into three major families: the delayed rectifier channels, the transient outward channels and the inward rectifier potassium channels. They are distinguished mainly by their responses to changes in membrane voltage. All voltage-gated potassium channels serve the same basic function, i.e. to create or stabilize a negative membrane potential, counteracting the depolarizing effects of channels that pass sodium or calcium ions (Adams and Nonner, 1989).

Delayed Rectifier Potassium Channel (K_V). The classical example of a delayed rectifier is the potassium conductance of the squid giant axon (Hodgkin and Huxley, 1952), which is activated with a brief delay following the onset of a membrane depolarization, and persists while the depolarization is maintained (i.e. it is non-inactivating). In squid axon, it

causes the quick repolarization that terminates the action potential (Adams and Nonner, 1989). The single channel conductance is quite variable ranging from 5-60 pS in different tissues. The K_V channel can be blocked by cesium, barium, millimolar intracellular tetraethylammonium (TEA) in most cells and external TEA or 4-aminopyridine (4-AP) in some cases (Rudy, 1988; Watson and Abbott, 1990).

The delayed rectifier channel contributes to the macroscopic outward potassium current observed in many cells including rabbit pulmonary arterial smooth muscle cells (Okabe et al., 1987), rat caudal artery and vein (Toro et al., 1986) or rabbit portal vein (Beech and Bolton, 1989a; Hume and Lebianc, 1989). A recent report has identified an outward potassium current in rat cerebral arteriole activated by depolarizing voltage steps beyond -20 mV (Bonnet et al., 1991). This current is sensitive to 4-AP, and is less sensitive to TEA. The current inactivates slowly and no decay is seen even with 3-second command pulses. A similar delayed rectifier potassium current has been identified from rat basilar arterial smooth muscle cells (Zhang et al., 1991a; Stockbridge et al., 1992). This current, which was recorded in the presence of low intra- and extracellular calcium, was sensitive to bath application of procaine or strychnine but not to TEA, was voltage-dependent, exhibited slow-inactivation, and slow sigmoidal activation kinetics.

Transient Outward Potassium Channel or A-Channel (K_A). This channel, like the delayed rectifier potassium channel, is activated by membrane depolarization, yet unlike the delayed rectifier, it decays (inactivates) spontaneously and rapidly while the depolarization is maintained. These channels have conductance from 5-38 pS and can be blocked by 4-AP (Adams and Nonner, 1989). Transient potassium currents were first described in molluscan neurons by Hagiwara et al. (1961). The term 'A current' was coined by Connor and Stevens (1971).

A rapidly developing, transient potassium current has been identified in rabbit portal vein, which is closely resembles the 'A current' (Beech and Bolton, 1989b). The activation and inactivation kinetics are fast and voltage-dependent. This current is blocked

completely by 4-AP or phencyclidine, but is insensitive to TEA, apamin, charybdotoxin, toxin-I or cromakalim. Similar 'A' currents have been observed in guinea-pig portal vein (Noack et al., 1990), rabbit pulmonary arterial smooth muscle cells (Okabe et al., 1987) and rabbit portal vein (Hume and Leblanc, 1989).

Inward Rectifier Potassium Channel (K_R). The channel is opened by large hyperpolarizations and passes sustained currents in the inward but not outward direction. This 'anomalous' rectification was first described in frog skeletal muscle (Katz, 1949). The channels which exhibit a conductance of approximately 20 pS can be blocked by cesium, barium or intracellular TEA (Rudy, 1988; Watson and Abbott, 1990).

In guinea-pig submucosal arterioles, Edwards and Hirst (1988) have demonstrated a potassium-selective inward rectification which supplies the dominant resting potassium conductance. A similar potassium current also exists in rat cerebral arteries (Edwards et al., 1988). In rat basilar arterial smooth muscle cells, hyperpolarizing potentials below -80 to -60 mV produce a rapidly activating inward current of small amplitude (Zhang et al., 1992a; Stockbridge et al., 1992), similar to that described by Hirst et al. (1986) from single electrode voltage-clamp recordings of rat middle cerebral arterioles.

CALCIUM-ACTIVATED POTASSIUM CHANNELS

In vascular smooth muscle, the potassium current activated by intracellular calcium is the predominant outward current (Singer and Walsh, 1984). Calcium-activated potassium channels couple calcium metabolism and membrane potential to potassium flux and membrane excitability. Three types of calcium-activated potassium channels have been described and these can be separated by their single channel conductance and their ease of blockade by a variety of pharmacological agents (Tomita, 1988; Cook, 1988a; Haylett and Jenkinson, 1990). However, the classification by conductance of these calcium-activated potassium channels is not entirely satisfactory. The conductances of the three types of calcium-activated potassium channels, namely large, intermediate or small, can range from 100-250, 18-60 or 4-14 pS (Castle et al., 1989) or > 150, 50-150 and < 50 pS respectively

(McManus, 1991). We take, in this section, McManus's (1991) defined conductance group as a standard for the classification because it is closer to the conductances (273, 180, 92pS) which have been identified in vascular smooth muscle (Inoue et al., 1985; 1986).

High Conductance Calcium-activated Potassium Channel (BK_{Ca}). BK_{Ca} is highly selective for potassium ions, activated by a low concentration of cytoplasmic calcium (0.01-1 μM), and has a very high conductance (200-300 pS in symmetrical high potassium concentration). The effect of increasing internal calcium concentration is considered mainly to increase the frequency of opening, i.e. to shorten the closed state and to prolong the open state (Tomita, 1988). The channels are also voltage-gated and are activated by membrane depolarization. There is evidence indicating that BK_{Ca} is also regulated by second messengers such as cAMP (Marty, 1989). BK_{Ca} can be blocked using extracellular TEA (submillimolar) or charybdotoxin (Rudy, 1988; Watson and Abbott, 1990).

BK_{Ca} channels are activated by intracellular calcium. The increase of intracellular calcium can be the result of either influx of extracellular calcium or release of calcium from intracellular stores. Removal of external calcium, application of calcium channel blockers (Ohya et al., 1987) or use of a pipette containing EGTA (5 mM) (Nakazawa et al., 1987; Yatani et al., 1987a) inhibit this component of the outward current. In addition to depolarization-induced BK_{Ca}, spontaneous transient outward potassium currents have been described in rabbit ear artery; this current was abolished by increasing the EGTA concentration in the pipette (Benham and Bolton, 1986). These currents are considered to be due to activation of potassium channels by calcium cyclically released from the intracellular store, since they can be activated transiently by caffeine and are abolished by ryanodine (Bolton and Peng Lim, 1989). Furthermore, histamine stimulates H_1 receptors and mobilizes calcium from an intracellular store, and thereby modulates a spontaneous transient outward current in rabbit ear artery (Neliat et al. 1989). The H_1 receptor agonist 2-(2-aminoethyl) pyridine (AEP) enhances BK_{Ca} channel activity by increasing

intracellular calcium concentration (Ishikawa et al., 1992).

BK_{Ca} channels have been reported in rat pancreatic vascular fragments (Stuenkel, 1989), rabbit portal vein (Inoue et al., 1985), rabbit or guinea-pig mesenteric arteries (Bolton et al., 1985; Benham et al., 1985; Benham et al., 1986), guinea-pig portal vein (Pfrunder and Kreye, 1991), rabbit aorta or pig coronary arteries (Gelband et al., 1989, 1990a,b), porcine or dog coronary arteries (Hu et al., 1991; Toro et al., 1991; Wilde and Lee, 1989) and human aorta (Bregestovski et al., 1988; Bolotina et al., 1991). A voltage-, calcium- and charybdotoxin-sensitive, large conductance (220 pS) potassium channel has been identified in rat basilar artery (Zhang et al., 1991a; Stockbridge et al., 1992).

Intermediate Conductance Calcium-activated Potassium Channel (IK_{Ca}). IK_{Ca} channel has been identified in human red blood cells, with a conductance of about 40 pS. This channel is highly calcium-sensitive but not very voltage dependent (Hamill, 1981). The channel is blocked by TEA (submillimolar), quinine or charybdotoxin (McManus, 1991; Watson and Abbott, 1990).

From cultured smooth muscle cells of rat aorta, a potassium channel activated by intracellular calcium, with a conductance of 135 pS, has been identified by single channel recording (Sadoshima et al., 1988a). Isoproterenol, which stimulates β-adrenoceptors, enhances the activity of this channel. This channel is modulated by cAMP-dependent phosphorylation (Sadoshima et al., 1988b). Similar conductances have been reported in vascular fragments of rat pancreas (91 pS; Stuenkel, 1989) and human mesenteric artery (80 pS; Trieschmann et al., 1988). Recently, a calcium-activated potassium channel has been identified in rat cerebral arteries (Wang and Mathers, 1991). This channel has a conductance of 92 pS and is dependent on voltage and intracellular calcium. It is blocked by intracellular application of TEA.

Small Conductance Calcium-activated Potassium Channel (SK_{Ca}). The SK_{Ca} channel exhibits a typical conductance of 6-14 pS and was first described in rat cultured skeletal muscle cells (Blatz and Magleby, 1987). These channels are selective for

potassium over sodium, activated by an increase in intracellular calcium, independent of membrane potential and can be blocked by apamin but not by TEA (Blatz and Magleby, 1987; McManus, 1991).

A channel with these characteristics has been described in rabbit portal vein (Inoue et al., 1985), although its conductance (92 pS) is too large for it to be classified as SK_{Ca}. It is not classified as an IK_{Ca} channel because it is insensitive to TEA. Similar calcium-activated potassium channels from rat aorta (Shoemaker and Worrell, 1991) and vascular fragments of rat pancreas (Stuenkel, 1989) have been identified; these also have a larger conductance of 55 pS or 43 pS respectively. But the SK_{Ca} in vascular fragments of rat pancreas is more sensitive to intracellular TEA than to calcium.

Extracellular Calcium-activated Potassium Channel (EK_{Ca}). An intermediate conductance calcium-activated potassium channel has been described in rabbit portal vein (Inoue et al., 1986) with a single channel conductance of approximately 180 pS. The channel is more sensitive to external calcium than internal calcium and more susceptible to internal than external TEA. This channel is open at resting potential which suggests a possible role in the maintenance of resting membrane potential. A similar potassium channel has been shown in porcine coronary artery, which has a conductance of 30 pS and is activated by extracellular calcium (Inoue et al., 1989).

RECEPTOR-COUPLED POTASSIUM CHANNELS

Two receptor gene superfamilies cover receptor-coupled ion channels (Pfaffinger and Siegelbaum, 1990). The first class is the "intrinsic sensor" type where the receptor and ion channel are part of a single macromolecular protein complex, such as nicotinic acetylcholine, GABA, glutamate, glycine receptors (Miller, 1988), and possibly K_{ATP} channels (Davies et al., 1991). The second class is the "remote sensor" type where that the receptor and channel are two distinct macromolecules, and their interactions are mediated by G proteins. The receptors which regulate potassium channels by G-proteins, include the adenosine A_1 receptor, α_{2A} adrenoceptor, dopamine D_2 receptor, $GABA_B$ receptor and

opioid μ, δ receptors (Watson and Abbott, 1990; Pfaffinger and Siegelbaum, 1990). The most studied receptor-coupled potassium channels include the M current (K_M), the 5-hydroxytryptamine inactivated ($K_{5\text{-}HT}$) potassium channel and the acetylcholine activated (K_{ACh}) potassium channel (Cook, 1988a; Watson and Abbott, 1990). Some of these channels have been reported in vascular smooth muscle.

Acetylcholine Activated Potassium Channels (K_{ACh}). There have been few studies of these channels in vascular smooth muscle. Acetylcholine augments calcium release from the sarcoplasmic reticulum, and the released calcium activates BK_{Ca} (130 pS) channels in guinea-pig coronary smooth muscle cells (Ganitkevich and Isenberg, 1990). In canine colonic smooth muscle, acetylcholine appears to suppress calcium-activated potassium current via a pertussis toxin-sensitive G protein linked to the muscarinic receptor (Cole and Sanders, 1989).

OTHER POTASSIUM CHANNELS

Potassium channels can be directly modulated by G proteins (Brown et al., 1991) and by various second messenger cascades activated by G proteins (Kaczmarek and Levitan, 1987; Belardetti and Siegelbaum, 1988). These second messengers include cAMP, intracellular calcium, products of phosphatidylinositol bisphosphate metabolism including inositol trisphosphate (IP_3) and diacylglycerol (DAG), and arachidonic acid (Pfaffinger and Siegelbaum, 1990). Other products, such as neurotransmitters, hormones, lipids and nucleotides also modulate calcium-activated potassium channels and possibly other types of channel also (Toro and Stefani, 1991).

G-Protein Activated Potassium Channels (K_G). The G proteins function as intermediates in transmembrane signaling pathways, receptors with effectors (Gilman, 1987). Some potassium channels, as effectors, can be directly activated by G-proteins, G_k (Brown and Birnbaumer, 1990; Brown et al., 1991). It has been proposed that the α subunits of G proteins can directly couple receptors to ionic channels, particularly potassium channels (Brown et al., 1991). The first demonstration of a direct regulation of

ionic channels by a specific G protein was made for atrial K_{ACh} channels (Yatani et al., 1987b). The G-protein directly activates the BK_{Ca} channel in pig coronary artery (Scornik et al., 1992).

cAMP-Sensitive Potassium Channels (K_{cAMP}). Receptors like the β-adrenergic receptor or the 5-HT receptor which are coupled to G_s promote dissociation and activation of the α subunit of G_s. This stimulates the activity of adenylyl cyclase to catalyse the conversion of ATP to cyclic AMP, which in turn activates cyclic AMP-dependent protein kinase (Blackshear et al., 1988). Agents which increase cAMP in smooth muscle include receptor agonists such as β-adrenoceptor agonists, adenosine and analogs, dopamine (acting through postsynaptic dopamine receptors-D_{1A}), glucagon, parathyroid hormone, vasoactive intestinal polypeptide and calcitonin gene-related peptide. Phosphodiesterase inhibitors and forskolin also elevate intracellular cAMP (Murray, 1990).

cAMP-dependent channels have been described in a variety of tissues (Kaczmarek and Strumwasser, 1984; Ewald et al., 1985). cAMP, isoprenaline, and forskolin increase the probability of opening of BK_{Ca} in cultured cells from rat aorta, indicating that cAMP-dependent phosphorylation may be involved in the activation of these channels (Sadoshima et al., 1988b). A direct action of cAMP on the sodium potassium pacemaker channel (i_f) in atria has been suggested by DiFrancesco and Tortora (1991).

cGMP-Sensitive Potassium Channels (K_{cGMP}). Many agents, like acetylcholine, 5-HT, A 23187, unsaturated fatty acids, histamine, bradykinin and substance P release endothelium-derived relaxing factor (EDRF) (nitric oxide) from endothelium (Rapoport and Murad, 1983), which activates cytoplasmic soluble guanylyl cyclase and increases cGMP, which might, in turn, regulate potassium channels (Goy, 1991). Nitrovasodilators release nitric oxide and elevate cGMP (Lincoln, 1989). Atrial natriuretic peptide (ANP) or ANP-like molecules may activate membrane guanylyl cyclase to produce the same effect.

cGMP has been shown to enhance the activity of BK_{Ca} in bovine aortic smooth muscle cells (Roy-Contancin et al., 1990). Several vasodilatory agents that increase

intracellular cGMP (nitroprusside, adenosine, and atrial natriuretic factor) have been reported to increase the activity of BK_{Ca} channels in bovine aortic smooth muscle cells (Williams et al., 1988). On the other hand, this group found that the modulation of potassium channels may be mediated by guanine nucleotides, such as GMP itself, rather than by cGMP, while adenine nucleotides were ineffective. Nitroglycerin activates a BK_{Ca} (300 pS) in cultured smooth muscle cells of porcine coronary artery by causing an increase of cGMP (Fujino et al., 1991). Bethanidine, nitroprusside and atrial natriuretic factor open a cGMP-sensitive potassium channel in aortic muscle (Bkaily, 1990). As can be seen, the available data in this area is confusing and sometimes contradictrory, and additional data is badly needed.

Potassium Channels Activated by metabolites of phosphatidylinositol (K_{PI}). Some receptors act through the unidentified "G_p" protein to activate phospholipase C, leading to the hydrolysis of phosphatidylinositol and release of inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Nishizuka, 1984).

IP_3 releases calcium from intracellular stores and this can lead to the opening of calcium-activated potassium channels (Higashida and Brown, 1986). IP_3 also increases outward potassium currents in rabbit portal vein (Ohya et al., 1988). DAG activates protein kinase C, and this process is accompanied by a translocation of the enzyme from the cytoplasm to the membrane (Nishizuka, 1988). DAG inhibits both calcium and voltage-dependent potassium currents in a variety of cells, including smooth muscle cells (Baraban et al., 1985), via the activation of protein kinase C. Protein kinase C specifically enhances a delayed rectifier potassium current in guinea-pig heart cells (Tohse et al., 1990).

Arachidonate-Sensitive Potassium Channels (K_{AA}). Arachidonic acid is a 20-carbon fatty acid, which can be released from membranes, either through the direct action of phospholipase A_2 or through the combined action of phospholipase C and diacylglycerol lipase (Pfaffinger and Siegelbaum, 1990). Arachidonic acid is metabolized through the cyclooxygenase pathway to prostaglandins, prostacyclins and thromboxanes, and through

the lipoxygenase pathway to hydroperoxyeicosatetraenoic acids and thus to leukotrienes and lipoxins.

The effects of arachidonic acid on potassium channels may result from a direct or indirect action (Ordway et al., 1991). Platelet-activating factor releases arachidonic acid metabolites, especially 5-lipoxygenase products, in cardiac myocytes. This causes a persistent stimulation of G_k resulting in a receptor-independent activation of the K_{ACh} channel by GTP (Nakajima et al., 1991). Evidence for the ability of arachidonic acid, as well as other fatty acids, to open potassium channels has been reported in rat atrial cells (Kim and Clapham, 1989) and toad stomach smooth muscle cells (Ordway et al., 1989). Similar results follow treatment with cholesterol or fatty acid have been observed in vascular smooth muscle (Bolotina et al., 1989; Bregestovski et al., 1989; Ordway et al., 1990; Katz et al., 1990; Kirber et al., 1990; Serebryakov et al., 1990).

ATP-Sensitive Potassium Channels (K_{ATP}). Potassium channels which close as $[ATP]_{in}$ increases have been described in cardiac muscle (Noma, 1983), pancreatic β-cells (Ashcroft et al., 1984), skeletal muscle (Spruce et al., 1985), neurons (Ashford et al., 1988) and vascular smooth muscle (Standen et al., 1989). Those in the last tissue have a higher conductance (135 pS) than other K_{ATP} channels which exhibit typical channel conductances between 40-80 pS (Rorsman and Trube, 1990) or 15-20 pS (Davies et al., 1991). The ATP-dependent potassium channels are normally closed at intracellular ATP concentrations in the physiological range, and opened when $[ATP]_{in}$ falls (Rorsman and Trube, 1990). The K_{ATP} channels exhibit slight inward rectification, and their probability of opening is weakly dependent on membrane potential (De Weille and Lazdunski, 1990). Sulfonylureas, especially glibenclamide, block K_{ATP} channels in pancreatic β-cells (Schmid-Antomarchi et al., 1987) and in vascular smooth muscle (Standen et al., 1989; Kovacs and Nelson, 1991). Results from [^{3}H] glibenclamide binding assays suggest that the sulfonylurea receptor is closely linked, if not identical, to the K_{ATP} channel itself (Schmid-Antomarchi et al., 1987; Kovacs and Nelson, 1991).

It has been suggested that the K_{ATP} channels form a family of channels differing to some extent between tissues in such things as conductance, ATP sensitivity, and sensitivity to blockade by sulfonylureas (Nelson et al., 1990a). The K_{ATP} channel in vascular smooth muscle is closed by ATP, opened by cromakalim and blocked by glibenclamide (Standen et al., 1989; Kovacs and Nelson, 1991). However, there are reports that BK_{Ca} channels, which have been identified from different vascular smooth muscles, are also sensitive to ATP (Gelband et al., 1990b), intracellular calcium (Silberberg and Van Breemen, 1990) and glibenclamide (Gelband et al., 1989; 1990a,b; Hu et al., 1990; Kajioka et al., 1990).

Sodium-Activated Potassium Channel (K_{Na}). This channel has a large conductance of 220 pS and is sensitive to TEA (Watson and Abbott, 1990). Sodium-activated potassium channels have been reported in heart myocytes and neuron preparations and the properties of this channel have recently been reviewed (Bader et al., 1990). Similar channels have been described in heart (Sanguinetti, 1990; Rodrigo and Chapman, 1990). Extracellular sodium indirectly controls potassium channel activity in porcine coronary artery cells (Kim and Hu, 1992).

Metabolite Modulated Potassium Channels (K_{MT}). BK_{Ca} channels are highly modulated by a large spectrum of metabolites (Toro and Stefani, 1991). Neurotransmitters, hormones, lipids, and nucleotides are capable of activating and/or inhibiting BK_{Ca} channels without a change in the intracellular calcium concentration. The modulation of BK_{Ca} channels in smooth muscle by these agents, such as substance P, histamine, acetylcholine, adrenergic agents, angiotensin II, arachidonic acid, fatty acid, cholesterol, and GMP, cGMP, GDP and GTP, has been detailed by Toro and Stefani (1991), and some of those agents are described elsewhere in this paper.

The examples in vascular smooth muscle are that angiotensin II directly inhibits BK_{Ca} (250 pS) from coronary smooth muscle incorporated into lipid bilayers (Toro et la., 1990) and outward potassium current from rabbit aorta (Bkaily et al., 1988). Vasopressin modulates several ion channels including potassium channels in rat aortic smooth muscle

cell line (Van Renterghem et al., 1988). In rabbit mesenteric artery, iodoacetic acid and dinitrophenol activate a voltage-insensitive potassium conductance, which is fully inhibited by glibenclamide (Silberberg and Van Breemen, 1992). They suggest that ATP-sensitive potassium channels underlie this conductance and hypoxia- induced vasodilation arises from the activation of K_{ATP} channels.

DRUGS WHICH MODULATE POTASSIUM CHANNELS

Potassium channel modulators include potassium channel agonists and antagonists. The effect of potassium channel agonists in cerebrovascular smooth muscle has been studied and their possible role against cerebral vasospasm tested. Needless to say, these agents can greatly affect the control of smooth muscle tone. The mechanism of the newly revealed *relaxant* effect of some potassium channel antagonists, namely sulfonylureas, remains to be elucidated. Potassium channel agonists include seven distinct groups of synthetic drugs, differing in their chemical and pharmacological characteristics, and some endogenous compounds (Brayden et al., 1991). Representative members of each group of synthetic drugs are nicorandil, pinacidil, cromakalim, minoxidil sulfate, diazoxide and RP 49356 (for details see Edwards and Weston, 1990b).

Even though these potassium channel agonists possess high potencies in the relaxation of vascular smooth muscle, it is still not clear that what type (or types) of potassium channel are modulated by these agonists. Since the history, pharmacological studies, structure-activity relationships, experimental effects and the potential clinical usage of these synthetic potassium channel agonists have been extensively reviewed (Cook, 1988a; Hamilton and Weston, 1989; Edwards and Weston, 1990a,b; Cook and Quast, 1990; Buckingham, 1990), in this review we will concentrate on the patch-clamp studies of drug action on specific potassium channels in vascular smooth muscle.

SYNTHETIC POTASSIUM CHANNEL AGONISTS

Nicorandil Group. The effect of the nicotinamide ester, nicorandil, on the coronary circulation in the dog was first described by Uchida et al. (1978). In anaesthetized

dogs, nicorandil produces a marked increase in coronary blood flow. The mechanism of relaxation was shown to be an increase in membrane potassium conductance (Furukawa et al., 1981) and also an activation of soluble guanylyl cyclase (Holzmann, 1983).

It is unclear which type (or types) of potassium channel in vascular smooth muscle is affected by nicorandil. Most patch-clamp studies on cardiac myocytes have demonstrated effects of nicorandil on several unidentified outward or inward potassium channels (Noda and Muramatsu, 1987; Satoh and Hashimoto, 1984; Habuchi et al., 1987) or ATP-dependent potassium channels (Hiraoka et al., 1989; Takano and Noma, 1990; Hamada et al., 1990). Nicorandil has no effect on calcium currents (Kakei et al., 1986; Iijima and Taira, 1986; Hiraoka et al., 1989).

In porcine coronary artery, nicorandil activates an potassium channel which is sensitive to extracellular calcium and which has a conductance of 30 pS (Inoue et al., 1989). In rat portal vein, a large conductance calcium-activated potassium channel (180 pS) is insensitive to nicorandil (500 μM), while a 10 pS calcium-activated potassium channel is nicorandil sensitive (Kajioka et al., 1990). Both of these currents are sensitive to intracellular calcium and to glibenclamide. The effect of nicorandil on smooth muscle cells from rat basilar artery has been studied by whole-cell and single-channel recordings. Nicorandil has no effect on the unitary conductance but has variable effects on the opening probability of the large conductance, calcium-activated potassium channel. Some of the experiments show a sustained increase of openings of the channel, while others only demonstrate an increase during the first few minutes exposure to the drug. With increased nicorandil concentration, the channel activity either increases, remains unchanged or even decreases. Nicorandil has no effect on the delayed rectifier or the calcium-activated potassium current in whole cell recordings (Zhang et al., 1991a).

Pinacidil Group. Even though pinacidil had been synthesized in the early 1970s, its ability to hyperpolarize smooth muscle was revealed only recently (Bray et al., 1987; Southerton et al., 1987; Cook et al., 1988b). Pinacidil produces dose-related reductions in

systemic blood pressure in rats, cats and dogs (Hamilton and Weston, 1989), and hyperpolarizations in rat aorta and portal vein (Bray et al., 1987; Southerton et al., 1987). Unlike nicorandil, pinacidil has no effect on guanylyl cyclase or adenylyl cyclase (Kauffman et al., 1986), but may inhibit calcium channels in guinea-pig mesenteric arteries (Nakashima et al., 1990), reduce chloride conductance in rat mesenteric arterioles (Videbaek et al., 1990) or attenuate the ryanodine-sensitive outward potassium current induced by calcium released from intracellular stores (Xiong, et al., 1991). There have been reports that pinacidil reduces the amplitude and duration of excitatory junction potentials in guinea-pig and rabbit blood vessels, but has no effects on labelled noradrenaline release (Nedergaard, 1989; Nakashima et al., 1990). Higher concentrations of pinacidil inhibit receptor-mediated, G-protein-coupled phosphatidyl inositol turnover (Anabuki et al., 1990). In addition to increasing potassium conductance, pinacidil produces intracellular calcium redistribution (Erne and Hermsmeyer, 1991).

Pinacidil can activate a variety of potassium channels including unidentified potassium channel (Iijima and Taira, 1987), ATP-sensitive potassium channels (Arena and Kass, 1989a,b; Escande et al., 1989; Tseng and Hoffman, 1990; Martin and Chinn, 1990; Findlay et al., 1989; Nakayama et al., 1990; Fan et al., 1990a,b) or calcium-activated transient outward current (Tseng and Hoffman, 1990) in cardiac myocytes. In vascular smooth muscle, it stimulates calcium-activated potassium channels (Hermsmeyer, 1989a,b; Hu et al., 1990) or delayed potassium current (Economos et al., 1990).

In rat azygous vein (Hermsmeyer, 1988a,b) or rat portal vein (Hu et al., 1990), pinacidil or its derivative P 1060 stimulates calcium-activated potassium channels which have conductances of 200 and 251 pS respectively. The open-state probability of the BK_{Ca} is increased by pinacidil and this action is blocked by glibenclamide and also by charybdotoxin (Hu et al., 1990). Pinacidil and cromakalim increase delayed outward potassium currents in aortic cells, which can be blocked by TEA and barium (Economos et al., 1990). A BK_{Ca}, but not delayed rectifier, is activated by pinacidil in smooth muscle cells

from rat basilar artery, but at a higher concentration than is observed in other system (Zhang et al., 1991a; Stockbridge et al., 1991). The whole-cell current and the single-channel opening probability are increased in the presence of pinacidil.

Cromakalim Group. Cromakalim lowers blood pressure in animal models by dilating blood vessels (Buckingham et al., 1986). This effect arises from hyperpolarization of vascular smooth muscle by the opening of potassium channels (Hamilton et al., 1986; Weir and Weston, 1986). Besides the ability to hyperpolarize the membrane, cromakalim possesses other actions. Cromakalim inhibits both the refilling and release of calcium from noradrenaline-sensitive intracellular stores in rabbit aorta (Bray et al., 1990; Bray et al., 1991), decreases calcium activity in central regions of myocytes and induces a shift of calcium distribution to primarily subsarcolemmal sites in rat caudal arteries (Erne and Hermsmeyer, 1991). Lemakalim, the active enantiomer of cromakalim, inhibits the refilling of an IP_3-sensitive calcium store in cultured smooth muscle cells derived from rabbit trachea (Chopra et al., 1990). Cromakalim also inhibits calcium currents in cells freshly isolated from rat portal vein, but has no effects on sodium inward current (Okabe et al., 1990).

Cromakalim resembles pinacidil in that most of the patch-clamp studies using vascular smooth muscle suggest that either delayed rectifiers (Beech and Bolton, 1989a; Economos et al., 1990), large conductance calcium-activated potassium channels (Kusano et al., 1987; Gelbend et al., 1989; 1990a,b; Klöckner et al., 1989; Hu et al., 1990; Okabe et al., 1990) or ATP-dependent potassium channels (Standen et al., 1989; Kovacs et al., 1991) are activated by cromakalim. In cardiac myocytes, unidentified (Osterrieder, 1988) or ATP-dependent potassium channels (Escande et al., 1988; Sanguinetti et al., 1988; Findlay et al., 1989; Pilsudski et al., 1990) are activated by cromakalim.

In vascular smooth muscle, the contribution of K_{ATP} and BK_{Ca} channels to the total potassium conductance, is still a matter for debate. Standen et al. (1989) have reported an ATP-dependent potassium channel in mesenteric artery smooth muscle from rabbit or rat.

They have concluded that the ATP-dependent potassium channel is the common pathway not only for cromakalim, pinacidil or RP 49356, but also for some neurotransmitters. A similar conclusion has been reached for the action of cromakalim in canine aortic smooth muscle (Kovacs et al., 1991). While in bovine pial, human mesenteric and pig coronary arteries, cromakalim activates the large conductance calcium-activated potassium channel (Trieschmann et al., 1988; Klöckner et al., 1989a). Similar results have been obtained from rabbit aorta (337 pS) (Gelband et al., 1989) and rat portal vein (Hu et al., 1990). These channels are blocked by both glibenclamide and charybdotoxin. These authors have been unable to demonstrate the existence of the ATP-dependent potassium current (Trieschmann et al., 1988; Gelband et al., 1989; Hu et al., 1990) reported by Standen et al. (1989). Effects of cromakalim on calcium-activated potassium channels have been reported in other vascular smooth muscle system (Kusano et al., 1987; Gelbend et al., 1990a,b; Okabe et al., 1990). In rat basilar artery smooth muscle cells, cromakalim activates a voltage- and calcium-activated, large conductance potassium channel, increases the whole-cell current and single-channel opening probability (Zhang et al., 1991a; Stockbridge et al., 1991).

Other Synthetic Potassium Channel Agonists. Both minoxidil sulphate and diazoxide, which are older drugs developed for other actions, have potassium channel opening properties (Bray, et al., 1988b; Meisheri et al., 1988; Leblanc et al., 1989; Newgreen et al., 1990). More recently, RP 49356 has been shown to be capable of opening potassium channels (Mondot et al., 1988). Niguldipine modulates both calcium currents and outward potassium currents (Klöckner and Isenberg, 1989; Klöckner et al., 1989).

There are few patch-clamp studies of minoxidil sulphate, RP 49356 and diazoxide in vascular smooth muscle. Minoxidil sulphate inhibits a calcium channel and activates a background potassium conductance in smooth muscle cells of rabbit portal vein (Leblanc et al., 1989). ATP-dependent potassium conductances, either 70 (Escande et al., 1989) or 66 pS (Thuringer and Escande, 1989), are activated by RP 49356 in guinea-pig cardiac myocytes. Most studies of diazoxide have concentrated either on β-cells or cultured insulin

secreting cell lines. Faivre and Findlay (1989) have reported that diazoxide modulates an ATP-dependent potassium channel in rat cardiac myocytes. Diazoxide increases the opening probability of a calcium-activated potassium channel (250 pS) in human mesenteric artery (Trieschmann et al., 1988). Both niguldipine and diazoxide increase BK_{Ca} currents in isolated vascular smooth muscle cell (Klöckner and Isenberg, 1989). Bay K 8644, a calcium-channel agonist, increases calcium-activated potassium channels in both aortic and heart muscle (Renaud et al., 1988), even though it is not usaully regarded as a potassium channel agonist.

ENDOGENOUS POTASSIUM CHANNEL AGONISTS

Endothelium-dependent relaxation of vascular smooth muscle in response to agents like acetylcholine, prostacyclin, calcitonin gene-related peptide, vasoactive intestinal polypeptide and substance P is accompanied by membrane hyperpolarization (Standen et al., 1989; Nelson et al., 1990b; Edwards and Weston, 1990a; Brayden et al., 1991). Recent studies have revealed that these agents possess properties of opening of potassium channels and relaxing of cerebrovascular smooth muscle cells.

POTASSIUM CHANNEL ANTAGONISTS-SULFONYLUREAS

Sulfonylureas, especially glibenclamide, have been known for several years to block K_{ATP} channels in pancreatic β-cells (Schmid-Antomarchi et al., 1987), in skeletal muscle (Spruce et al., 1985) and in vascular smooth muscle (Standen et al., 1989; Kovacs and Nelson, 1991). Glibenclamide inhibits calcium-activated potassium channels (Gelband et al., 1989; 1990a,b; Hu et al., 1990) but not delayed rectifier potassium channels (Escande et al., 1988; but see Beech and Bolton, 1989a) nor calcium currents (Schmid-Antomarchi et al., 1987). Despite this, glibenclamide and other sulfonylureas have been found to *relax* vascular smooth muscle in variety of tissues by mechanisms which do not appear to involve direct effects on potassium conductance.

CEREBROVASCULAR EFFECTS OF POTASSIUM CHANNEL MODULATORS

The significance of pharmacological control of vascular potassium channels is considerable, but in the cerebral circulation the possibility of promoting the relaxation of constricted vessels is particularly important in cerebrovascular spasm. This condition is an intense delayed vasoconstriction which occurs several days after rupture of an intracranial aneurysm and is accompanied by significant morbidity and mortality. There are currently no drugs which have been unequivocally shown to reverse established vasospasm in humans or to abort the development of vasospasm, although the calcium antagonists may have such effects (Cook, 1984), and there is good evidence that they decrease the severity of the neurological deficit associated with vasospasm, at least in some patients, possibly by other mechanisms than by causing vasodilatation. The discovery of the potassium channel activators has suggested that these drugs might prove useful, and the possible application of these drugs in cerebral vasospasm is the subject of the remainder of this review.

Oxyhemoglobin is now believed the probable etiological reason for the development of cerebral vasospasm (Macdonald and Weir, 1991). Oxyhemoglobin releases calcium from intracellular IP_3-sensitive stores (Vollrath et al., 1990), enhances calcium-activated potassium channels (Steele et al., 1990), or depolarizes the membrane or increases the ionic conductance (Fujiwara and Kuriyama, 1984). The damage to arterial smooth muscle induced by oxyhemoglobin may be mediated by generation of free radicals, production of eicosanoids and release of endothelin from endothelium cells (Macdonald and Weir, 1991). Many potassium channel modulators have been tested for their ability to prevent or reverse cerebral vasospasm. Most of the studies have been carried out in *in vitro* or *in vivo* experimental situations, while little clinical data about the effect of these drugs are available.

SYNTHETIC POTASSIUM CHANNEL AGONISTS

Nicorandil Group. Most of the clinical studies of nicorandil have been in relation to management of angina pectoris, although it also possesses some actions in control of coronary vasospasm. Several clinical studies suggest that nicorandil selectively dilates the

coronary vasculature, without influence on other hemodynamic factors such as heart rate, blood pressure and cardiac output (Sakai et al., 1983; Kinoshita and Sakai, 1990). There do not appear to be any clinical reports of effects of nicorandil on cerebral vasospasm, which is of course a completely different phenomenon than coronary spasm.

Since nicorandil modifies neither the membrane potential nor the membrane resistance of guinea-pig basilar arteries, it was suggested that this artery lacks of a potassium channel which is sensitive to nicorandil (Fujiwara and Kuriyama, 1983). However, Harder et al. (1987) have observed, in isolated basilar artery from a canine model of subarachnoid hemorrhage, that there is a marked membrane depolarization and an enhanced electrical spike activity in spastic artery which results from reduction in resting potassium conductance. Nicorandil hyperpolarizes the smooth muscle cells and increases internal diameter, and infusion of nicorandil in anesthetized dogs subjected to subarachnoid hemorrhage reversed by 50% the reduction in basilar artery diameter. Similar observations have been reported by Yamada et al. (1989) and by Sakakura and Waga. (1990). Nicorandil causes prolonged relaxation of canine basilar artery contracted by prostaglandin $F_{2\alpha}$, U 46619, endothelin, 3,4-diaminopyridine (a potassium channel blocker) or potassium chloride, although these effects may also be related to the increase of cGMP produced by nicorandil. Intravenous injection of nicorandil cannot prevent the development of vasospasm in a dog model, while intrathecal injection of nicorandil produces a marked dilatation of the spastic basilar artery (Yamada et al.,1989).

The mechanism of action of nicorandil in cerebral arteries has been studied by blockade of ATP-dependent potassium channels with glibenclamide (Schmid-Antomarchi et al., 1987), or inhibition of soluble guanylyl cyclase with methylene blue (Greutter et al., 1981). The effect of nicorandil was blocked by methylene blue but not by glibenclamide (Zhang et al., 1992b), which suggested that the relaxant effect of nicorandil in canine cerebral arteries is mediated mainly by activation of guanylyl cyclase rather than by opening of potassium channels. This result is in agreement with a patch-clamp study using

rat basilar artery smooth muscle cells which has shown that nicorandil does not increase potassium currents while cromakalim and pinacidil do (Zhang et al., 1991a; Stockbridge et al., 1991). On the other hand, in rat cerebral arteries, the effects of nicorandil were competitively inhibited by glibenclamide and TEA, but not by tolbutamide (Ksoll et al., 1991; Parsons et al., 1990).

Oxyhemoglobin released from clotted blood in the subarachnoid space depolarizes and contracts cerebral arterial smooth muscle. Nicorandil, which opens potassium channels, activates guanylyl cyclase, and relaxes the contractions induced by eicosanoids, endothelin and oxyhemoglobin, is a hybrid molecule with potassium channel opening properties associated with the pyridyl moiety and guanylyl cyclase activation generated by the nitroxy side-chain. Even though a tenfold greater concentration of nicorandil is required to block TEA-induced contractions in post-subarachnoid hemorrhage arteries than in control rabbit basilar arteries (Young and Pickard, 1990), the two *in vivo* studies (Harder et al., 1987; Yamada et al., 1989) highlight its potential role in the treatment of cerebral vasospasm.

Pinacidil Group. A recent multicentre clinical trial has shown that pinacidil provides an effective therapeutic effect in the management of hypertension (Goldberg, 1988). It produces a decrease in blood pressure and increase in heart rate, effects sometimes associated with a typical vasodilator headache. Oedema is also a prominent side-effect of pinacidil, as a consequence of the reduced blood pressure and stimulation of renal compensatory mechanisms. Both oedema and headache are reduced by concurrent treatment with a thiazide diuretic.

The relaxant effect of pinacidil in canine arteries pre-contracted by prostaglandin $F_{2\alpha}$ has been reported and compared with those of nifedipine (Toda et al., 1985). Pinacidil is most active in the coronary artery followed by renal, mesenteric, basilar and middle cerebral arteries. Nifedipine on the other hand, is most active on the basilar artery followed by renal, mesenteric and coronary arteries. In canine cerebral arteries, nimodipine achieves

its ED_{50} at lower concentrations than that of pinacidil (Zhang et al., 1992b). On the contrary, the potency of pinacidil in dilating feline pial arteries is about 100 times higher than that of nimodipine (Wahl, 1989). Thus, the observed effects of pinacidil and calcium channel antagonists in smooth muscles may vary according to the methods used, experimental species and the agonists used to precontract the tissue (O'Donnell et al., 1991).

The action of pinacidil in cerebral arteries is inhibited by tolbutamide (Wahl, 1989; but see Ksoll et al., 1991) and glibenclamide (Zhang et al., 1992b; Ksoll et al., 1991) but not by methylene blue (Zhang et al., 1992b; but see Parsons et al., 1990). This suggests that pinacidil exerts its effect by opening potassium channels. However, as suggested previously, the specificity of glibenclamide for ATP-dependent potassium channels is not established beyond doubt (Zhang et al., 1991b; 1992b), so the type of potassium channels in cerebral arteries which is activated by pinacidil has not been firmly established (Zhang et al., 1991a; Stockbridge et al., 1991). The relaxant effect of pinacidil in rat cerebral arteries is competitively inhibited by TEA but not by tolbutamide (Ksoll et al., 1991). Some other mechanisms have been suggested for the actions of pinacidil. Pinacidil may produce vasodilatation due to interference with the transmembrane influx of calcium into smooth muscle evoked by receptor stimulation. Decreased release of calcium from intracellular stores or increased sequestration of calcium may also be involved (Toda et al., 1985). Recent observations in rat basilar artery show that both methylene blue and glibenclamide inhibit the relaxant effects of nicorandil, pinacidil and cromakalim, suggesting that pinacidil, like nicorandil, activates soluble guanylyl cyclase in that preparation (Parsons et al., 1990).

Until recently, pinacidil has not been directly tested in models of vasospasm. Even though pinacidil is less effective in relaxing contraction induced by prostaglandin $F_{2\alpha}$ in canine cerebral arteries than nimodipine, preliminary observations in our laboratory suggest that pinacidil (10^{-5} M), may be as effective as nimodipine (10^{-5} M), in relaxing the contraction induced by oxyhemoglobin (10^{-5} M) in canine cerebral arteries. Pre-application

of pinacidil attenuates the contractile responses to oxyhemoglobin but not that to KCl (60 mM), while pre-application of nimodipine inhibits both. More studies are required to elucidate the potential therapeutic usage of pinacidil in management of vasospasm.

Cromakalim Group. Clinical investigations have revealed that cromakalim produces a lowering of systolic and diastolic blood pressure in supine hypertensive patients (Singer et al., 1989). Some incidence of headache was reported, particularly at the highest dose, but oedema was not seen, which may result from actions of cromakalim to increase renal blood flow and glomerular filtration rate (Gluck et al., 1987).

Using the tracer microspheres method, Hof et al. (1988) have reported that cromakalim preferentially dilates the coronary, gastrointestinal, and cerebral vessels but not those of the kidneys or skeletal muscle. The relaxant effects of cromakalim have been compared with nimodipine. In rabbit basilar artery, only the first component of the contraction induced by 5-HT is cromakalim-sensitive whereas nimodipine depresses both components of the response (Cain and Nicholson, 1989). Cromakalim produces dose-dependent relaxation of rabbit basilar arteries precontracted with 5-HT, KCl and neuropeptide Y, but produces small relaxations (< 50%) of rabbit thoracic aorta precontracted with noradrenaline, KCl or 5-HT (Grant and O'Hara, 1990). Cromakalim inhibits receptor-mediated contraction at low concentration and myogenic tone at high concentrations in rat cerebral arteries (Nagao et al., 1991).

The relaxant responses to cromakalim or lemakalim are competitively antagonized by glibenclamide in cerebral arteries from dog (Masuzawa et al., 1990; Zhang et al., 1992b), rat (Ksoll et al., 1991) and rabbit or cat (Parsons et al, 1991a), suggesting that the drug opens potassium channels. Cromakalim increases the opening probability of a large-conductance calcium-activated potassium channel and the whole-cell current in rat basilar arterial smooth muscle cells (Zhang et al., 1991a; Stockbridge et al., 1991). Although the effects of cromakalim are not inhibited by tolbutamide, they are noncompetitively inhibited by TEA (Ksoll et al., 1991). Other than opening of potassium channels, cromakalim has

been suggested to have other functions, because it relaxes contraction induced by high KCl (Nagao et al., 1991) and the effect of cromakalim has been inhibited by methylene blue (Parsons et al., 1990), suggesting a mechanism involving guanylyl cyclase.

Since cerebral smooth muscle is more sensitive to the effect of cromakalim and since its actions are not limited only to membrane hyperpolarization, cromakalim or lemakalim, like nicorandil, may prove to be ideal candidates for the treatment of cerebral ischemia, especially for cerebral vasospasm. More *in vivo* experiments using models of vasospasm need to be conducted.

Other Synthetic Potassium Channel Agonists. Both diazoxide and minoxidil sulfate are vasodilators used in management of hypertension. Diazoxide has been tested against the vasospasm which occurs after subarachnoid hemorrhage by Heros et al. (1976) who found that intracisternal injection of diazoxide in dogs and intracarotid injection in monkeys failed to relieve cerebral vasospasm, but produced hypotension and increased mortality, which suggests that diazoxide lacks selectivity for cerebral arteries. Low concentrations of RP 49356, a new potassium channel opener, nicorandil or cromakalim, constrict rabbit basilar arteries partially contracted by 5-HT and KCl, while higher concentrations of these drugs cause relaxation (Young and Pickard, 1990). Minoxidil, but not the bioactive minoxidil sulfate, has been tested on canine basilar artery and produced little relaxation even at high concentrations (Zhang et al., 1990). No data about cerebrovascular effects of minoxidil sulfate and niguldipine are available.

ENDOGENOUS POTASSIUM CHANNEL AGONISTS

Hyperpolarization can be evoked by a variety of potassium channel agonists including the synthetically-derived agents mentioned above, as well as by endogenous compounds such as acetylcholine, calcitonin gene-related peptide, vasoactive intestinal polypeptide and endothelial factors. Like synthetic potassium channel agonists, these endogenous agents also hyperpolarize the membrane, close voltage-dependent calcium channels and lead to vasodilation (Brayden et al., 1991; Nelson et al., 1990) as outlined in

an earlier section.

Acetylcholine (ACh) and Endothelium-Derived Hyperpolarizing Factor (EDHF). Acetylcholine (ACh) relaxes smooth muscle by causing release of both endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor (EDHF) in guinea-pig basilar artery (Nishiye et al., 1989). EDRF has been identified as nitric oxide which is believed to induce relaxation by increasing the level of cyclic GMP in the cytosol (Furchgott and Jothianandan, 1983; Moncada et al., 1991). Hemoglobin and methylene blue inhibit the relaxation caused by EDRF, by binding the nitric oxide or by inhibition of guanylyl cyclase respectively (Martin et al., 1985; Hongo et al., 1988; Gruetter et al., 1981). The chemical nature of EDHF is unclear. It has been suggested that EDHF differs from nitric oxide (Taylor and Weston, 1988; Moncada et al., 1991) in that it is not bound by hemoglobin, nor inhibited by methylene blue. However, nitric oxide has been shown to cause hyperpolarization by increasing potassium efflux in some arteries (Tare et al., 1990) and this effect can be blocked by the potassium channel antagonist 4-AP (Elliott et al., 1991). EDHF is not a cylooxygenase product, since its response is not affected by indomethacin (Chen et al., 1988).

The mechanism by which EDHF opens potassium channels is not known, although ACh mediated membrane hyperpolarization and relaxation are antagonized by glibenclamide in vascular smooth muscle, suggesting the possible involvement of K_{ATP} channels (Standen et al., 1989; Nelson et al., 1990b; but see Hamada et al., 1990; McPherson and Angus, 1991; Elliott et al., 1991). ACh also modulates calcium-activated potassium channels (Toro and Stefani, 1991). Like other potassium channel agonists, EDHF-induced hyperpolarization serves to reduce the opening probability of voltage-dependent ion channels involved in the excitatory responses of many agonists. ACh hyperpolarizes and thus relaxes rabbit middle cerebral arteries and these effects can be blocked by glibenclamide or barium chloride (Brayden, 1990). The relaxation and hyperpolarization of feline cerebral arteries induced by ACh are not associated with cGMP

(Brayden and Wellman, 1989). On the other hand, ACh-induced relaxation in rabbit middle cerebral arteries is not inhibited by glibenclamide nor by ouabain, an inhibitor of Na, K-ATPase, while it is inhibited by NG-nitro-L-arginine, an inhibitor of nitric oxide synthesis (Parsons et al., 1991b). Relaxation of canine basilar artery by ACh and nitric oxide is blocked by 4-AP but not by glibenclamide (Elliott et al., 1991). These contradictory results may merely represent the differences between tissues or experimental methods, but they do call into question the specificity of glibenclamide. The issue of the similarities between EDRF and EDHF remains to be resolved.

The relaxant actions of ACh, histamine and A23187 are attenuated both by potassium channel blockers and by high concentrations of methylene blue (Adeagbo and Malik, 1990). EDRF produces the long lasting relaxation and EDHF may contribute to an initial transient relaxation (Komori et al., 1988; Keef and Bowen, 1989). Hemoglobin inhibits the relaxation induced by EDRF but only slightly inhibits the hyperpolarization induced by EDHF in guinea-pig basilar artery (Nishiye et al., 1989). Since EDHF initiates mechanical inhibition, hyperpolarizes the membrane and thus indirectly closes any voltage-dependent calcium channels, it does contribute significantly to endothelium-dependent relaxation (Taylor and Weston, 1988). Relaxant effect of ACh on isolated cerebral arteries has been extensively investigated (Toda, 1974, 1979; Young et al., 1981; Brayden, 1990; Elliott et al., 1991), but its lack of selectivity and rapid metabolism makes it an unlikely candidate for clinical use in vasospasm.

Prostacyclin. Prostacyclin is released from vascular endothelium and produces relaxation by activation of adenylyl cyclase (Gryglewski et al., 1988). Prostacyclin or its analog, iloprost, which also exerts its vasodilatatory effect either by increasing of intracellular concentration cAMP (Murray, 1990) or by opening of potassium channels (Siegel et al., 1989a,b; 1990), relaxes cerebral arteries from different species (Chapleau and White, 1979; Parsons and Whalley, 1989; Zhang et al., 1992d). cAMP may modulate potassium channels by activation of cAMP-dependent protein kinases (Osterrieder et al.,

1982; Walsh and Kass, 1988) or by direct activation (DiFrancesco and Tortora, 1991).

During subarachnoid hemorrhage, oxyhemoglobin which is released from the blood clot, may exert its effect by generation of eicosanoids. Prostaglandins $F_{2\alpha}$, E_2, D_2 and thromboxane A_2, are increased and prostacyclin is decreased during the development of vasospasm (Brandt et al., 1983; Weir, 1987). The possible role of prostacyclin and its analogues in management of vasospasm has been reviewed (Wilkins, 1986). Iloprost dilates the spastic arteries from a canine model of subarachnoid hemorrhage and relaxes the contraction induced by oxyhemoglobin (Zhang et al., 1992d). Iloprost does not relax the contraction induced by high concentrations of potassium and it is possible that this arises from an action of iloprost on potassium channels (Zhang et al., 1992d). Topical application or intrathecal injection of iloprost reverses vasospasm in feline or canine models of subarachnoid hemorrhage (Egemen et al., 1988; Egemen et al., 1990). A clinical report also provided encouraging results in treatment of vasospasm following subarachnoid hemorrhage (Stanworth et al., 1988).

Calcitonin Gene-Related Peptide (CGRP). CGRP is a 37-amino-acid peptide produced by alternative processing of messenger RNA from the calcitonin gene (Emeson et al., 1989). CGRP is one of the most potent vasodilators known. It has been found in perivascular nerves (Kawasaki et al., 1988) and has been detected in the blood of normal patients (Morris et al., 1984). The mechanism by which it dilates arteries is not known, but may be related to an increase of cAMP (Edvinsson et al., 1985).

Nelson et al. (1990b) have recently reported that CGRP hyperpolarizes and relaxes rabbit mesenteric smooth muscle by opening of potassium channels and this action is antagonized by glibenclamide and barium, suggesting the involvement of the K_{ATP} channel, a view which is consistent with results from cell-free single-channel studies. The mechanism by which CGRP opens potassium channels is not clear, nor is the hyperpolarization-independent component of the dilation produced by CGRP, which is glibenclamide unsensitive, well undersood.

CGRP relaxes rabbit basilar artery precontracted by 5-HT, but no relaxation occurred when the artery was precontracted by KCl. No relaxation occurs in rabbit common carotid artery (Hongo et al., 1989). The effect of CGRP is not endothelium-dependent, at least in cerebral arteries (Hanko et al., 1985; Edvinsson et al., 1985; Hongo et al., 1989), and CGRP is equally effective in relaxing control and spastic arteries. Improvement of cerebral ischaemia has been reported in a dog model of vasospasm (Nozaki et al., 1990), and in patients having delayed cerebral ischaemia (Johnston et al., 1990). But the mechanism of these effects, and value in patients with ruptured intracranial aneurysm remains to be determined.

Vasoactive Intestinal Polypeptide (VIP). VIP is a potent vasodilator, which was first isolated from porcine small intestine and is widely distributed in perivascular nerves (Gibbins et al., 1984). VIP belongs to a family of peptides including secretin, glucagon, corticotropin-releasing factor, gastric inhibitory peptide, growth hormone-releasing factor and the structurally-related peptide, histidine isoleucine (Mione et al., 1990). Several lines of evidence imply that VIP is a cerebrovascular neurotransmitter responsible for vasodilation (Lee et al., 1984; Brayden and Bevan, 1986). The vasodilation produced by VIP is independent of endothelium (Lee et al., 1984; Fahrenkrug, 1989), and exogenously applied VIP hyperpolarizes isolated cerebral arteries. This response is abolished by glibenclamide, suggesting involvement of potassium channels (Standen et al., 1989). VIP also causes elevation of the intracellular concentration of cyclic AMP in cerebral arteries (Edvinsson et al., 1985).

Immunohistochemical methods have revealed that the density of VIP and substance P in perivascular nerve fibers in canine basilar and middle cerebral arteries is markedly decreased after subarachnoid hemorrhage (Uemura et al., 1987), and the concentration of VIP in cerebrospinal fluid is reduced in humans with recent cerebral infarction (Wikkelsö et la., 1985). Subarachnoid hemorrhage also impairs the postsynaptic mechanism of VIP-induced dilatation of rabbit basilar arteries (Tsukahara et al., 1989). The vasorelaxant

actions of VIP and CGRP have been tested in experimental subarachnoid hemorrhage (Nozaki et al., 1990). The results suggest that VIP-induced relaxations of the major cerebral arteries are more or less impaired one to two weeks after subarachnoid hemorrhage. CGRP has more potent vasorelaxant action than VIP in both spastic and normal arteries.

Substance P (SP). SP is an undecapeptide belonging to the tachykinin family which also includes neurokinin A, neurokinin B and neuropeptide K (Mione et al., 1990). SP receptors have been localized on the endothelial cell surface (Stephenson and Summers, 1987), and relaxations mediated by these receptors may be coupled to the formation of cyclic GMP (Bolton and Clapp, 1986). SP, as well as ACh, bradykinin, ATP and A23187 release EDRF from endothelium, which produces vasodilatation (Angus and Cocks, 1989), but SP also directly modulates potassium channels in neurons (Rudy, 1988). SP promotes the simultaneous activation of multiple calcium-activated potassium channels in colonic smooth muscle by elevation of intracellular calcium concentration (Toro and Stefani, 1991). In vascular smooth muscle, the relaxation induced by SP is accompanied by endothelium-dependent hyperpolarization (Beny and Brunet, 1988; Beny et al., 1986).

Like CGRP and VIP, the relaxant effect of SP has been studied in both normal and spastic cerebral arteries (Saito et al., 1991; Kawakami et al., 1991). SP relaxes cerebral arteries from humans, cats, and pigs (Mejia et al., 1988). Its effect is endothelium dependent, while those of CGRP and VIP are endothelium independent (Mione et al., 1990). The SP-induced relaxation is weaker than that of CGRP and VIP, and it is not associated with cAMP accumulation (Edvinsson et al., 1985). SP-containing nerve fibers on the cerebral arteries could constitute the sensory link in a reflex arc system involved in the development of vasospasm (Delgado-Zygmunt et al., 1990). Depletion of SP-containing sensory nerves to the cerebral arteries with capsaicin prior to subarachnoid hemorrhage, and intrathecal or intracisternal administration of spantide, a SP antagonist, prevents the development of cerebral vasospasm in a rat model of vasospasm (Delgado-Zygmunt et al., 1990).

Atrial Natriuretic Peptide (ANP), Adenosine and Nitrovasodilators. Second messengers, as cAMP or cGMP, modulate potassium channels, especially BK_{Ca} in vascular smooth muscle (Sadoshima et al., 1988b; Roy-Contancin et al., 1990). The relation between cAMP, generated by prostacyclin, VIP or CGRP, and ATP-dependent potassium channels has been described above. Several other vasodilatory agents that increase intracellular cGMP, like SP (Toro and Nefoni, 1991), and nitroprusside, adenosine, or atrial natriuretic factor (Williams et al., 1988), have been reported enhance the activity of BK_{Ca} channels in smooth muscle cells.

The modulation of potassium channels in bovine aortic smooth muscle may involve guanine nucleotides, especially GMP, rather than cGMP, while adenine nucleotides are ineffective (Williams et al., 1988). Nitroglycerin activates a BK_{Ca} (300 pS) in cultured smooth muscle cells of porcine coronary artery by increasing cGMP (Fujino et al., 1991). The nitrovasodilators, such as sodium nitroprusside and glyceryl trinitrate hyperpolarize and relax uterine arteries which are depolarized and constricted by phenylephrine (Parkington et al., 1989). While the calcium antagonist, nimodipine, relxes contractions produced either by high concentration of potassium or by eicosanoids, glyceryl trinitrate is not very effective in relaxing contractions elicited by depolarization with high concentration of potassium, suggests that it may have some effect on potassium channel opening (Zhang et al., 1992d). Activation of an adenosine receptor, by adenosine and its analogues, hyperpolarizes bovine coronary smooth muscle cells (Sabouni et al., 1989) and rabbit ear or mesenteric arteries (Zhang et al., 1989). The K_{ATP} channel may be directly coupled to adenosine receptors via G proteins in heart (Brown et al., 1991). The dilation of coronary arteries by adenosine is inhibited by glibenclamide (Daut et al., 1990) but the adenosine-induced potassium current is not sensitive to this agent (Hamada et al., 1990). Adenosine induces large conductance calcium-activated potassium channels by increasing GMP (Williams et al., 1988).

The effects of nitrovasodilators in models of vasospasm have been wildly reported.

Intravenous nitroglycerin (Kistler et al., 1979; Frazee et al., 1981) and nitroprusside (Hirsh, 1980) increase the diameter of the constricted arteries without marked decrease of blood pressure (Kistler et al., 1979). Continuous intravenous infusion of nitroglycerin for 14 days in patients with ruptured cerebral aneurysms prevented severe vasospasm and improved the surgical results (Goto et al., 1990). Similar results have been shown in another report (Doi et al., 1990).

ANP is a powerful vasorelaxant, especially in renal arteries (Winquist and Hintze, 1990). Specific receptor sites for ANP have been identified in blood vessels (Chabrier et al., 1987; Winquist, 1985) and one of these receptors is an integral part of particulate guanylyl cyclase (Murad, 1986), where ANP causes endothelium-independent vasodilatation (Yanagisawa et al., 1987). The studies of ANP in vasospasm are at present not directly related to its vasodilating effect, but rather with hyponatremia which accompanied vasospasm (Diringer et al., 1988; Rosenfeld et al., 1989).

Adenosine compounds have vasodilatatory actions, which may accompanied by an increase of cAMP or cGMP (Collis, 1989) and have been used in the studies of vasospasm (Wilkins, 1986). Topical application of dibutyryl cAMP reverses the vasoconstriction induced by prostaglandin $F_{2\alpha}$ in cat basilar artery (Peterson et al., 1975) and reverses acute vasospasm in monkey cerebral arteries (Peterson et al., 1973). Combined treatment with ATP and prostacyclin prevents the degeneration of the arterial wall after experimental subarachnoid, and produces immediate vasodilatation (Haciyakupoglu et al., 1985).

POTASSIUM CHANNEL ANTAGONISTS

The recent explosion of research on potassium channel toxins has greatly advanced our understanding of potassium channels (Cook, 1988a; Moczydlowski et al., 1988; Castle et al., 1989; Strong, 1990). Closing of potassium channels will result in membrane depolarization (Fujiwara and Kuriyama, 1983) which in turn may contract smooth muscle. Potassium channel antagonists, such as 3,4-diaminopyridine, 4-aminopyridine or charybdotoxin, produce phasic contractions in human, swine and monkey coronary arteries

and in canine basilar, carotid, renal and femoral arteries (Uchida et al., 1986; Jones et al., 1990). The contractions continue for more than 11 hours and result in perinuclear vacuolization, a characteristic change of coronary vasospasm (Uchida et al., 1986). Other more selective antagonists, such as glibenclamide which is uasully classified as an ATP-dependent potassium channel antagonist are also available, and would be expected to cause contraction and thus exacerbate vasospasm.

Glibenclamide. Glibenclamide is a sulfonylurea which is used clinically as an oral hypoglycemic agent. It was first synthesized in 1966 and used against type II diabetes (Prendergast, 1984). The mechanism of action of glibenclamide involves blockade of the ATP-dependent potassium channel in pancreatic β-cells (Schmid-Antomarchi et al., 1987). This results in depolarization of membrane potential, calcium influx and insulin secretion. The ability of glibenclamide to block ATP-dependent potassium channels has also been demonstrated in cardiac myocytes (Sanguinetti et al., 1988) and in vascular smooth muscle (Standen et al., 1989). Glibenclamide depolarizes rat small mesenteric artery (McPherson and Angus, 1991), blocks or attenuates the vasorelaxant effects of potassium channel openers cromakalim and pinacidil (Buckingham et al., 1989; Cavero et al., 1989; Winquist et al., 1989; Zhang et al., 1992b), leads to the suggestion that these potassium channel agonists relax smooth muscle by opening of the ATP-dependent potassium channel.

Curiously, however, glibenclamide relaxes arterial rings contracted with prostaglandins or oxyhemoglobin (Zhang et al., 1991b,c; 1992c), and it has other inhibitory actions as well. It competitively and selectively inhibits the contractile response of canine coronary artery to a thromboxane A_2 analog, U 46619 (Cocks et al., 1990) and relaxes the contractions produced by prostaglandin $F_{2\alpha}$, D_2 and E_2 in canine cerebral arteries by a mechanism which is not endothelium dependent nor are they dependent on soluble guanylyl cyclase (Zhang et al., 1991b). Glibenclamide also relaxes the contractions induced by KCl (Chappell et al., 1991) and NA (Yoshitake et al., 1991) suggesting that this compound may have some effects on calcium channels, although we have not observed any

effects on these agonists in our system (Zhang et al., 1992c). Glibenclamide enhances prostacyclin release from rat thoracic aorta (El Tahir et al., 1986).

Other Sulfonylureas. Since the discovery of a sulfonamide which causes hypoglycemia in 1944, thousands of sulfonylurea-like compounds have been synthesized in an attempt to find more potent, more effective agents. A number of sulfonylurea derivatives are currently marketed as oral hypoglycemic agents, including the first generation drugs tolbutamide, chlorpropamide, acetohexamide and tolazamide, and the second generation agents such as glipizide, gliclazide and glibenclamide (Jackson and Bressler, 1981). The cardiovascular effects of these drugs have been reviewed (Jackson and Bressler, 1981; Huupponen, 1987).

In vascular smooth muscle, tolbutamide has been reported to decrease hypoxia- and diazoxide-induced contraction of rat pulmonary artery (Robertson et al., 1989) and NA-induced contraction of dog femoral artery (Lee et al., 1988). Preincubation of the rat aorta with gliclazide, acetohexamide, glibornuride and chlorpropamide enhance release of prostacyclin (El Tahir et al., 1986). Reversal of early diabetic microangiopathy has been reported with glipizide (Prendergast, 1984). Patch-clamp studies have revealed that these sulfonylureas possess the same action as glibenclamide of closing the ATP-dependent potassium channel in pancreatic β-cells (Schmid-Antomarchi et al., 1987). The potencies of blocking the ATP-dependent potassium channel by these agents are closely related to their potencies to reduce blood glucose (Schmid-Antomarchi et al., 1987), and their inhibitory effects on the contraction induced by prostaglandin $F_{2\alpha}$ (Zhang et al., unpublished observation). The densities of sulfonylurea binding sites in rat brain are decreased after transient ischemia (Mourre et al., 1990). No data is available concerning their actions in cerebrovasculature.

SUMMARY

Our knowledge of potassium conductance is based extensively on studies of nerve and skeletal or cardiac muscle, but there is increasing evidence that vascular smooth muscle

cells contain potassium channels which have some similarity to those observed in other tissues. Most known types of potassium channel have been discovered in at least one or two vascular preparations, but it is seems likely that in cerebrovascular smooth muscle, most of the potassium current is carried by calcium-activated potassium channels (Zhang et al., 1991a; Stockbridge et al., 1992; Wang and Mathers, 1991) with delayed rectifiers (Bonnet et al., 1991; Zhang et al., 1991a; Stockbridge et al., 1992) and inward rectifiers also present (Stockbridge et al., 1992; Zhang et al., 1992a; Hirst et al., 1986). In peripheral vessels there is a significant ATP-dependent potassium conductance (Standen et al., 1989), but so far this has not unequivocally been demonstrated in the cerebral vasculature. Indeed much of the evidence for the existence of K_{ATP} depends on sensitivity to glibenclamide, which has been widely believed to be selective for ATP-dependent potassium channels. Recent evidence, however, has suggested that this agent can also block the calcium-activated potassium channel (Gelband et al., 1989, 1990a,1990b; Hu et al., 1990; Kajioka et al., 1990) and the delayed rectifier (Beech and Bolton, 1989a). The issue is further complicated by the observation that the vascular calcium-activated potassium channel is sensitive not only to intracellular calcium, but also to ATP (Gelband et al., 19901). It seems intrinsically improbable that the calcium-activated potassium channel is responsible for hyperpolarization and relaxation since the intracellular calcium concentrations under these circumstances are likely to be very low (Hamilton and Weston, 1989). Thus the exact nature of the channel or channels which underlines the hyperpolarization accompanying relaxation is still unclear (Table II-1).

We do know that agents like pinacidil or cromakalim are capable of causing relaxation of precontracted cerebrovascular smooth muscle preparations principally by increasing the activity of the potassium channel, and that this effect is antagonized by glibenclamide but not by charybdotoxin nor by apamin (Hamilton and Weston, 1989; Quast and Cook, 1989). A variety of other agents which affect potassium conductance, can also cause relaxation by other mechanisms. Nicorandil, which is usually classified as a

potassium channel agonist, probably causes relaxation by activation of soluble guanylyl cyclase, and, as such, closely resemble the better-known vasodilators such as nitroglycerine. A variety of other receptor agonists including both peptides and small molecules, have some direct or indirect activity at potassium channels, although their principal mechanism of action may involve other mechanisms, such as promoting an increase in intracellular cyclic AMP. One of the more curious observations is that glibenclamide, which blocks the action of the potassium channel agonists in a variety of vascular preparations, can itself cause relaxation, probably by a mechanism which is unrelated to changes in potassium conductance.

In cerebrovascular spasm, there is good evidence that at least in the early phase of the condition, the problems arise from contraction of vascular smooth muscle rather than mechanical obstruction arising from anatomical changes in the vessel wall (Findlay et al., 1991). This observation, together with the finding that that cerebrovascular smooth muscle cells are depolarized during vasospasm (Varsos et al., 1983; Harder et al., 1987) suggests that the potassium channel agonists may be clinically useful. Indeed, in a variety of models of vasospasm, particularly using isolated tissues, these agents have been shown to cause relaxation of arteries constricted by a variety of spasmogens, including both oxyhemoglobin and eicosanoids both of which have convincingly been associated with vasospasm. Known compounds which cause relaxation primarily by opening potassium channels are not, however, selective for the cerebral circulation, and it may thus be necessary either to develop a delivery system which will present the drug directly to the arteries in spasm, or to devise new compounds with higher selectivity. Available data does suggest that the potassium channels in cerebral arteries may differ from those in peripheral arteries, and thus it may be feasible to design such compounds. There is little in the way of clinical evidence at present to support the use of the potassium channel agonists in patients, but it is probable that such trials will soon be available.

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Table II-1. Summary of patch-clamp studies on potassium channel agonists in vascular tissue

Tissue	Drug	K_{ATP}	K_{Ca}	Other	pS	Reference
Aorta smooth muscle						
Bovine	C		+		200	Kusano et al., 1987
Canine	C	+				Kovacs et al., 1991
Rabbit	C		+		337	Gelband et al., 1989, 1990
	C		+		200	Kusano et al., 1987
	C,P			+		Economos et al., 1990
Arterial smooth muscle						
Human mesenteric	C,D		+		250	Trieschmann et al 1988
Human mesenteric	C,D,Ni		+		180	Klöckner et al., 1989a
Bovine pial	C,D,Ni		+		180	Klöckner et al., 1989a
Porcine coronary	C,D,Ni		+		180	Klöckner et al., 1989a
Porcine coronary	N		+		30	Inoue et al., 1989
Porcine coronary	C		+*		300	Gelband et al., 1990b
Rabbit mesenteric	C	+			135	Standen et al., 1989
Rat mesenteric	C	+			135	Standen et al., 1989
Rat basilar	N		+?		220	Zhang et al., 1991a
	P,C		+		220	Stockbridge et al., 1991
Venous smooth muscle						
Rabbit portal	C			+		Beech & Bolton, 1989
	M			+		Leblanc et al., 1989
Rat portal	N		+*		10	Kajioka et al., 1990
	C,P*		+		251	Hu et al., 1990
	C		+			Okabe et al., 1990
Rat azygous	P		+		200	Hermsmeyer, 1988a,b

N = nicorandil, C = cromakalim, P = pinacidil, M = minoxidil sulfate, D = diazoxide, Ni = niguldipine, P* = P 1060 (analog of pinacidil), Other = delayed rectifier or other unidentified potassium channels. +* = K_{Ca} which is also sensitive to ATP.

CHAPTER THREE
POTASSIUM CURRENTS OF RAT BASILAR ARTERY SMOOTH MUSCLE CELLS

SUMMARY

Primary isolates of smooth muscle cells from the basilar artery of the rat were studied using whole-cell and single-channel patch-clamp techniques. Two distinct potassium currents were characterized. With low intracellular calcium, depolarization above 0 mV elicited an outward current of a few hundred pA (at +120 mV) with sigmoidal onset and little inactivation during 1.25 s steps. This current was reduced by bath application of 1 mM procaine or 1 mM strychnine, but not by 500 nM charybdotoxin. These are characteristics of the delayed rectifier potassium current in other preparations. With higher intracellular calcium, depolarization above 0 mV elicited a non-inactivating potassium current of several nA (at +120 mV). This current persisted in the presence of 1 mM procaine or strychnine but was reduced by bath application of 100 nM charybdotoxin. In whole-cell recordings in which intracellular calcium was unbuffered with EGTA, spontaneous transient outward currents were manifest and displayed voltage dependence and tail currents similar to the calcium-activated current. The spontaneous transient current and the calcium-activated current had similar sensitivity to charybdotoxin. Cell-free membrane patches contained 1 or more channels of 220 pS (in symmetrical potassium) with similar voltage and calcium dependence. These are characteristics of the large conductance calcium-activated potassium current in other preparations.

INTRODUCTION

The ionic currents underlying the electrical activity of arterial smooth muscle cells have been studied in several preparations. Arteries of the cerebral circulation have

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been studied by intracellular recordings (Harder, 1980; Harder et al., 1987; Waters and Harder, 1985) and single electrode voltage-clamp techniques (Hirst et al., 1986), but relatively little has been verified with whole-cell or single-channel patch-clamp techniques (Bonnet et la., 1991).

A number of unique features of cerebral arteries led us to undertake a more thorough analysis of the electrical properties of these cells. Cerebral arteries differ morphologically (Zervas et al., 1982), electrically (Harder, 1980) and pharmacologically (Hill et al., 1986; Katusic and Shepherd, 1988) from arteries in other areas. Furthermore, following subarachnoid hemorrhage, cerebral arteries react with spasm to blood in the extravascular space (Toda et al., 1980). This cerebral vasospasm is a serious complication to victims of cerebral aneurysm rupture. The cause of vasospasm is unclear and its therapy and prevention is nonspecific and incompletely effective.

There is evidence that alterations in potassium conductance are involved in vasospasm (Harder et al., 1987; Steele et al., 1991). Whether this relationship is causal or not, potassium channels are targets for a number of pharmacological agents which may have a role in the prevention or reversal of smooth muscle cell depolarization, contraction and the establishment of the vasospastic state. As a preliminary to exploring the utility of these drugs, the potassium currents in cerebral artery smooth muscle cells were characterized. Isolated smooth muscle cells were studied because of superior space clamp conditions, improvements in access of solutions to internal and external membrane surfaces, and because isolated cells react with the prime candidate spasmogen in cerebral vasospasm (Steele et al., 1991). Primary isolated cells (Steele et al., 1991) were studied to minimize de-differentiation of smooth muscle cells (Absher et al., 1989).

Materials and Methods

Cell isolation and recording techniques were similar to those reported previously (Steele et al., 1991).

Isolation of Rat Basilar Artery Vascular Smooth Muscle Cells. Sprague-

Dawley rats were anesthetized with halothane and decapitated. The basilar arteries were removed to a medium consisting of (mM): NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, HEPES 10, penicillin (100 U/ml) and streptomycin (0.1 g/l). Arteries were then cleaned of connective tissue and small side branches. The basilar artery was moved to a medium in which the $CaCl_2$ was reduced to 0.2 mM and to which were added collagenase (Type II, 0.5 g/l), elastase (0.5 g/l), hyaluronidase (Type IV- S, 0.5 g/l), and deoxyribonuclease I (0.1 g/l). The arteries were cut into 0.2 mm rings and incubated for 1 h at room temperature. The rings were transferred to fresh solution containing $CaCl_2$ (0.2 mM), trypsin inhibitor (0.5 g/l) and deoxyribonuclease I (0.1 g/l) and then triturated gently. Cells were plated on glass cover slips and stored at 4°C in saline containing $CaCl_2$ (0.8 mM) and essentially fatty-acid-free bovine serum albumin (2 g/l).

Isolated cells stained positive for α-actin retained the ability to contract in response to KCl, caffeine, serotonin, angiotensin II, prostaglandins $F_{2\alpha}$ and E_1 and oxyhemoglobin and (Steele et al., 1991).

Whole-cell Patch-clamp Technique. Whole-cell currents were recorded using standard techniques (Hamill et al., 1981) and an Axopatch 1C patch-clamp amplifier (Axon Instruments). The current data were analog-filtered at 5 KHZ (4-pole Bessel). No digital filtering was done. The membrane potential and current signals were stored on a laboratory computer. Under all recording conditions, currents and potentials across the membrane were described with the usual convention. The normal bath solution for the whole-cell recordings was (mM): NaCl 130, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8 (for recording the calcium-activated potassium current) or 0 (for recording the delayed rectifier potassium current), HEPES 10, glucose 5.2 and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with (mM): KCl 139, $MgCl_2$ 0.5, $CaCl_2$ 0.1 (for the calcium-activated potassium current) or 0 (for the delayed rectifier potassium current), EGTA 0.09 (for the calcium-activated potassium current) or EDTA 10 (for the delayed rectifier potassium current), HEPES 10, glucose 10 and the pH was adjusted to 7.4 with KOH. The free calcium concentration in

the high calcium pipette solution was estimated to be 10 μM. Bath solution changes were made with a syringe pump (Sage model 351), modified for simultaneous withdrawal and injection. All experiments were done at room temperature (19 - 22 °C). Outward currents were eliminated when cesium replaced potassium in the pipette solution (Steele et al., 1991).

Leakage conductance was determined by applying small, hyperpolarizing voltage steps. None of the records shown were leakage corrected. Cell capacitance measurements were done by integrating the current recorded in response to hyperpolarizing voltage steps insufficient to evoke an inwardly rectifying current and calculating the capacitance from $C = \int Idt/V$. Electrode resistances were 1-2 MΩ, resulting in up to 4 mV series resistance error when studying the largest outward currents. Seal resistances of over 1 GΩ were obtained routinely. Series resistance compensation was not employed. Under whole-cell patch-clamp conditions, the cells had input resistances of 5-10 GΩ and capacitances of 20-30 pF.

Capacitance compensation was never done during recording, but was done during data analysis by subtraction of the scaled response to small command potentials. Imperfections in cancellation of capacitive transients are visible in some figures. These imperfections prevented study of fast delayed rectifier tail currents but had negligible effect on much slower tails of calcium-activated potassium currents.

Analysis of voltage homogeneity. Smooth muscle cells isolated from the rat basilar artery were not more than 150 μm long, 5 μm wide and 2 μm thick, with a cross section perhaps equivalent to a cylinder of 3 μm diameter. Infolding and the presence of numerous caveoli (Mulvaney, 1986) increase the surface area of the cell probably several times that suggested by these measurements, to perhaps 3000 μm^2 or $3x10^{-5}$ cm^2. Measured cell capacitances of 20-30 pF, at 1 $\mu F/cm^2$, would correspond to 2000-3000 μm^2. The cytoplasmic resistivity (R_a) in smooth muscle is about 200 Ωcm (Tomita, 1969). Typical whole-cell currents in these cells are consistent with a peak membrane conductance of about 2 nS, corresponding to a specific membrane resistivity (R_m) of $1.5x10^4$ Ωcm^2. From

Hodgkin & Rushton (Hodgkin and Rushton, 1946), the length constant would be $\sqrt{(aRm)/(2Ra)}$ (where a is the radius) = 750 μm or 5 times the length of a fully relaxed cell. Short cable theory (Jack et al., 1975) can be used to show that a voltage-clamp pulse applied to the middle of such a cell and which produced the largest observed current would still be expected to control the distal end of the cell to within > 99% at steady state and > 93% at 50 μs. A similar conclusion was reached by Toro et al. (Toro et al., 1986).

Single Channel Recording. Single channel current recordings were conducted at room temperature in the inside-out configuration (Hamill et al., 1981). The bath (intracellular) solution had the following composition (mM): KCl 140, $CaCl_2$ 0.008, $MgCl_2$ 0.5, HEPES 10, EGTA 0.09, glucose 10 and the pH was adjusted to 7.4 with KOH. The free calcium concentration in this solution was estimated to be 2 nM. The pipette medium (extracellular solution) contained (mM): KCl 140, $CaCl_2$ 0.1, $MgCl_2$ 0.5, HEPES 10, glucose 10, pH 7.4 with KOH. Single channel recordings were made with the Axopatch 1C amplifier filtering at 5 KHZ and stored digitally on video tape (Neurocorder model 384 digitizer and Sony SL-700 video recorder). On playback, data was redigitized at 10 kHz with a DT-2841-F (Data Translation) analog-digital converter on an AT-compatible computer. Amplitude histograms were made from contiguous segments of data 120 s long. Software written by the authors detected channel openings by crossing of 50% or 150% thresholds between histogram peaks. No digital filtering was done.

RESULTS

One component of the outward current was evident in response to depolarizing potentials in the low calcium pipette solution (n = 18). Characteristics of this current are shown in Fig. III-1. The current was never greater than a few hundred pA at +120 mV (Fig. III-1B). The current was activated by potentials above -20 to 0 mV (Fig. III-1D and 1E). The onset kinetics were clearly sigmoidal in shape and the onset was faster at more depolarized potentials (Fig. III-1B). A simplex curve fitting algorithm minimizing square error was used to fit the current in response to a +72 mV step (Fig. III-1F) to an exponential

relaxation raised to a power: $I = A [1 - e^{t/\tau}]^n$. The method fit a power of 3.6. The current showed no significant decay or inactivation during a step to +120 mV for 1.25 s (Fig. III-1C). Tail currents decayed too quickly at hyperpolarizing potentials to permit accurate separation from the capacitive transient, so it was not possible to measure a reversal potential directly. The steady-state currents and a reversal potential of -82 mV computed from the Nernst equation were used to determine the conductance as a function of potential (Fig. III-1E).

The delayed rectifier potassium conductance decreased during whole-cell recordings, usually over a period of 30 min or more. No attempt was made to find a pipette constituent to prolong the lifetime of this current.

When the calcium concentration in the pipette was high, a second and usually larger component of outward current was observed in response to voltage steps above -30 mV. With the EGTA-buffered pipette solution, the responses to voltage commands from -100 to +60 mV are shown in Fig. III-2 (HIGH PIPETTE CA^{2+}). The onset kinetics for this current and its voltage-dependence were similar to those of the delayed rectifier current, but several pharmacological features served to distinguish them.

The current seen with high internal calcium tended to increase in amplitude during recording, as seen in comparing control and washout in Figs. III-3 and III-4. The increase was greater when EGTA was omitted from the pipette solution. This increase was unrelated to drug treatment.

Procaine (Fig. III-2) and strychnine (Fig. III-3) were relatively selective blockers of the current evoked by depolarization in the low calcium pipette solution. About 60% of the current was blocked by procaine (1 mM, n = 2). About 90% of this current was blocked by strychnine (1 mM, n = 2). There was no effect of 1 mM procaine or strychnine on the outward current evoked by depolarization in the high intracellular calcium medium.

Charybdotoxin (100 nM, n = 3) blocked the calcium-activated potassium current (Figs. III-4) but 500 nM charybdotoxin (n = 2) had no effects on the delayed rectifier

current. The effect on the calcium-activated current was fully reversible.

Spontaneous transient outward currents appeared when EGTA and calcium were omitted from the internal solution (Fig. III-5). When the holding potential was -20 mV, no spontaneous events were observed. With strong depolarizing holding potentials, the currents were as much as 1 nA and lasted tens to several hundred ms. Delayed rectifier tail currents decayed much more quickly than did those of the calcium-activated potassium current. Depolarizing steps with the unbuffered intracellular medium produced currents with irregular shapes, suggesting the superposition of one or more spontaneous components. Tail currents occurring when these spontaneous currents fell at the end of the test potential always decayed slowly, consistent with the spontaneous currents resulting from opening of calcium-activated potassium channels. Spontaneous transient outward currents were most readily seen in cells which were relaxed and phase bright.

Single channel recordings from inside-out membrane patches usually contained more than one large conductance ion channel. Fig. III-6 shows its characteristics. Channel current and open probability as a function of holding potential are shown for an inside-out patch recorded with a free calcium concentration in the bath of 0.1 μM (n = 3). A line fit to the slope of the current-voltage plot (Fig. III-6A) had a slope of 218 pS (symmetrical 140 mM potassium). Channel open probability (Fig. III-6B), based on two channels in the patch, was computed from 120 s of data at each holding potential. Raw data from this patch at holding potentials of 0-60 mV are shown in Fig. III-6C.

The calcium sensitivity of the open probability has been assessed in inside-out membrane patches recorded for 120 s at 10 kHz (n = 5). At a holding potential of -70 mV and a free calcium concentration estimated to be 20 nM, the channel open probability was 0.01. When the free calcium concentration was raised to 200 nM, the open probability rose to 0.03.

Channel openings corresponding to a conductance of about 40 pS appeared at low frequency in some recordings. These channels were not characterized further.

The calcium-activated potassium current in whole-cell recordings tended not to run down as quickly as the delayed rectifier current. It was not unusual for the calcium-activated current to increase over a 20-30 min recording.

In cells studied within 24 h following dissociation, a small inward current was elicited by command potentials below -80 mV. This current had rapid activation kinetics and little or no inactivation within 100 ms. This current was usually less than 50 pA at -120 mV. The ionic dependence of this current has not been established.

DISCUSSION

Using whole cell and single channel patch-clamp techniques, two potassium current components have been distinguished in smooth muscle cells freshly dissociated from the rat basilar artery.

There is a calcium-activated potassium current. Its activation requires intracellular calcium levels above 10 nM and membrane potentials above -20 to 0 mV or intracellular calcium levels above 100 nM and membrane potentials above -60 mV. This current was relatively insensitive to 1 mM procaine and strychnine, but was blocked by 100 nM charybdotoxin (Miller et al., 1985). Cell-free membrane patches typically contained multiple channels of about 220 pS with voltage and calcium sensitivity similar to the macroscopic current. In general the characteristics of the whole-cell and single channel currents were similar to those mediated by maxi-K channels in other arterial and other smooth muscle cells (Benham et al., 1986; Bolton et al., 1985; Toro et al., 1986; Tomita, 1988).

One implication of the calcium sensitivity of these potassium channels is that the free calcium concentration in relaxed smooth muscle cells (in which the delayed rectifier is the principal outward current) must be less than 100 nM.

When the intracellular calcium concentration was poorly buffered, spontaneous transient outward currents were observed. These currents had characteristics similar to the calcium-activated potassium current in this preparation and to spontaneous transient

outward currents in smooth muscle cells of rabbit ear artery (Benham and Bolton, 1986) and rabbit ileum (Ohya et al., 1987). Elongated and relaxed cells were more likely to show spontaneous outward currents than were cells which were contracted following dissociation. On the basis of this observation, it is suggested that these spontaneous currents represent effects of normal cycling of intracellular calcium.

Under conditions in which the intracellular calcium was low, a smaller potassium current was distinguished. It had similar voltage dependence to the calcium-activated current but had slower and more sigmoidal activation kinetics. This current was relatively sensitive to bath application of procaine or strychnine. Single channels responsible for the macroscopic delayed rectifier current were not identified. Characteristics of this current were quite similar to that of the delayed rectifier potassium current first described in the squid axon (Hodgkin and Huxley, 1952) and in nonvascular smooth muscle (Tomita, 1988). This current is similar to the potassium current identified in freshly dissociated smooth muscle cells from cerebral arteries of the cat (Bonnet et al., 1991). The study of cat cerebral arterial smooth muscle cells failed to find evidence for a calcium-activated potassium conductance. The arteries used in that study were not specified. The present study was conducted on the basilar artery alone dissociated after having been cleaned of side branches. Electrophysiological studies of intact cerebral arteries show evidence of regional differences (e. g., Hirst et al., 1986) and these differences warrant systematic study in isolated cells.

Hyperpolarizing potentials below -80 to -60 mV produced a rapidly activating inward current of small amplitude. This current has not been seen in most investigations of arterial smooth muscle (e.g., Toro et al., 1986) but was described by Hirst et al. (1986) from single electrode voltage- clamp recordings of rat middle cerebral arterioles. This current was not manifest in all cells in the present study, but was seen more often in cells studied within 24 h of dissociation than in those studied later. The differences may therefore be technical rather than species-related or tissue-related.

Smooth muscle cells from the rat basilar artery contained other currents as well. Calcium currents in these cells will be the subject of a subsequent report. We found no evidence for a sodium current, which is consistent with smooth muscle of other sources (Tomita, 1988). In cell-free membrane patches studied in symmetric chloride near 0 mV, there was no evidence for the large conductance chloride channel found in rat aorta smooth muscle (Sadoshima et al., 1989).

There have been several previous studies of the electrical properties of cerebral artery smooth muscle pertinent to cerebral vasospasm. Waters and Harder (1985) used microelectrode current-clamp recording techniques to show that the resting potential of smooth muscle cells of cat cerebral arteries exposed to clot was more positive than that of cells of control arteries. Ouabain produced a roughly equivalent depolarization of cells in control and clot-exposed arteries. Harder et al. (1987) used similar techniques to show that the slope of the relationship between the potassium concentration in the bath and the resting membrane potential was reduced following subarachnoid clot placement around the dog basilar artery. They concluded that potassium conductance was reduced by exposure to clot. Steele et al. (1991) showed that smooth muscle cells isolated from rat basilar arteries and exposed to oxyhemoglobin evidenced an increased calcium-activated potassium conductance, presumably because of a rise in intracellular calcium. They suggested that the results of Harder et al. (1987) were influenced by decreased electrical coupling of smooth muscle cells, also a consequence of raised intracellular calcium.

Harder et al. (1987) showed a partial reversal of clot-induced spasm by nicorandil, a potassium channel agonist. This is a significant result regardless of the details of the mechanism leading to cerebral vasospasm. We are currently surveying potassium channel agonists and attempting to determine the channel types they affect in isolated basilar artery smooth muscle cells.

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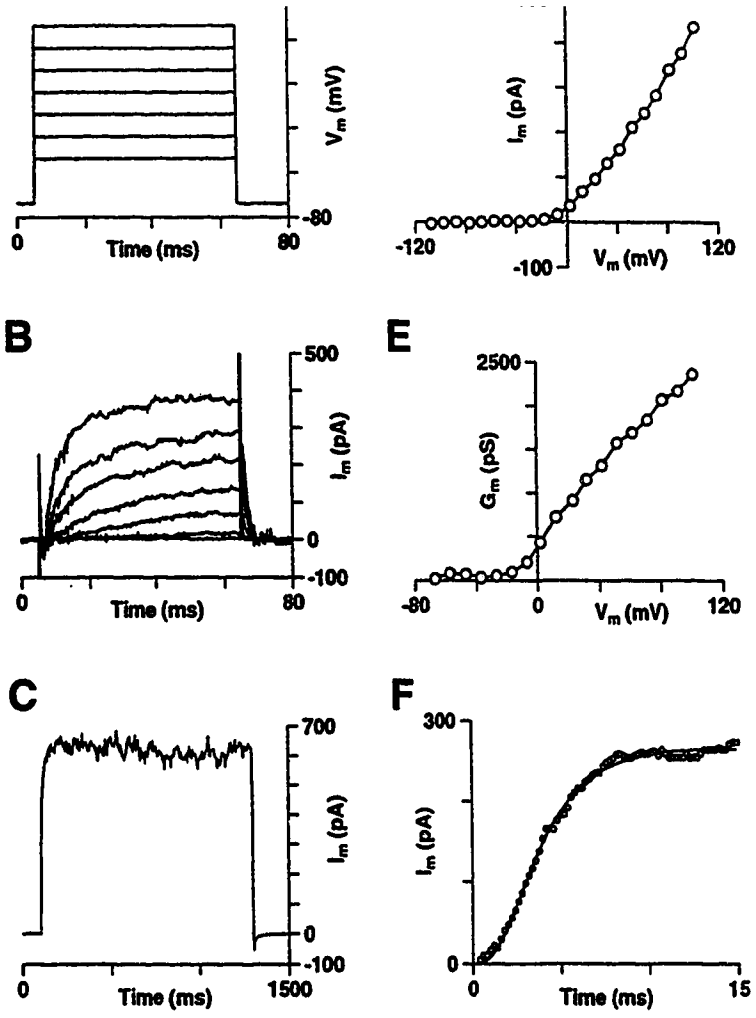

Fig. III-1. Whole-cell delayed rectifier potassium current recorded in the standard
l and external solutions. The holding potential was -67 mV. A. Voltage-clamp
nds, 7 sweeps (-27, -7, +13, +33, +53, +73 and +93 mV) superimposed. B. Outward
s recorded in response to voltage commands shown in A. C. Average current
d in response to three 1250 ms steps to +120 mV. D. Peak current versus command
al from experiment shown in A and B. E. Peak conductance versus command
al based on data in D and a reversal potential of -82 mV calculated from the Nernst
al for potassium. F. Current recorded in response to command step to +72 mV was
l exponential relaxation raised to the power of 3.6.

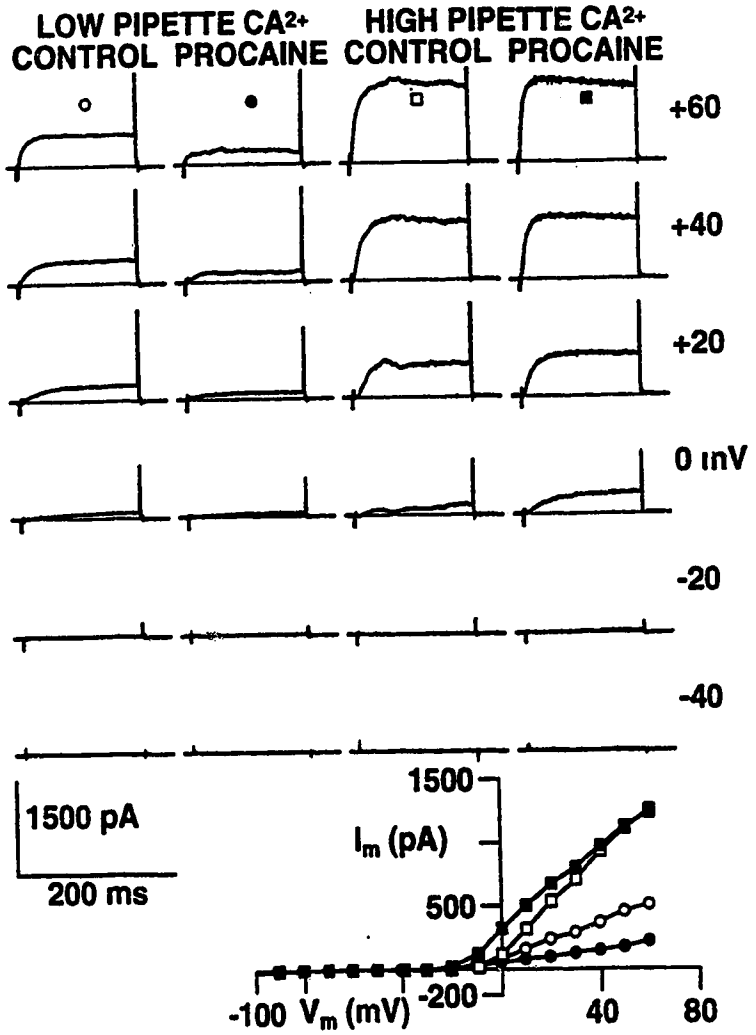

Fig. III-2. TOP: Effect of procaine (1 mM, columns 2 and 4) on currents recorded in the standard internal solution (columns 1 and 2) or high internal calcium solution (columns 3 and 4). The zero-current level is marked. The holding potential ws -60 mV. Voltage-clamp command potentials are shown on the extreme right. BOTTOM: Peak current versus voltage for data shown above.

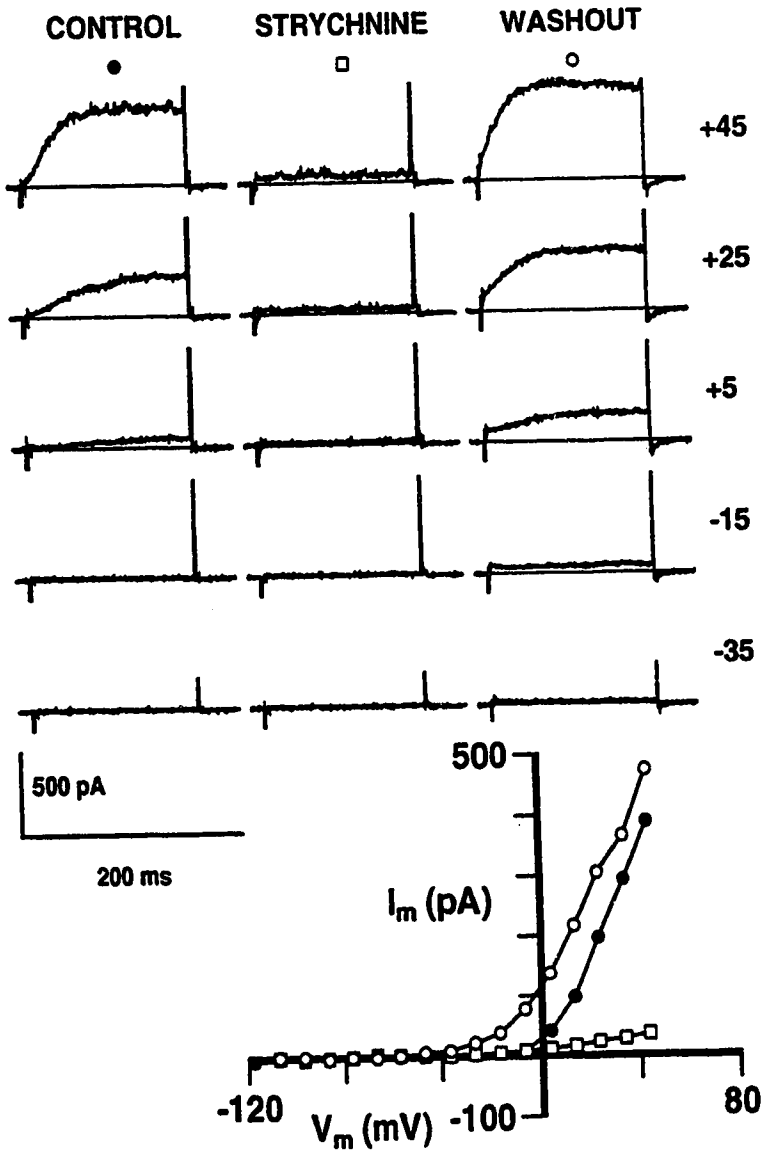

Fig. III-3. TOP: Effect of strychnine (1 mM, column 2) on currents recorded in the low calcium solution. The zero-current level is marked. The holding potential was -60 mV. Voltage-clamp command potentials are shown on the extreme right. BOTTOM: Peak current versus voltage for data shown above.

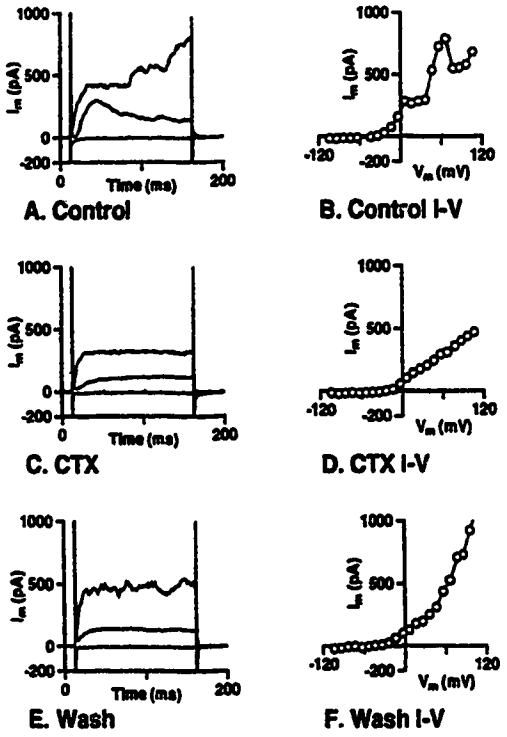

Fig. III-4. The effect of charybdotoxin on whole-cell patch-clamp recordings. Charybdotoxin (100 nM; C and D) blocked most of the outward current, including the spontaneous transient component. The remaining component had the amplitude and slow sigmoidal kinetics associated with the delayed rectifier current. The holding potential was -60 mV. Voltage commands for the three traces shown in panels A, C and E were -100, +10 and +60 mV. Spontaneous transient outward currents in the control sweeps and in the wash sweeps contributed to the irregularities in the peak current-voltage plots.

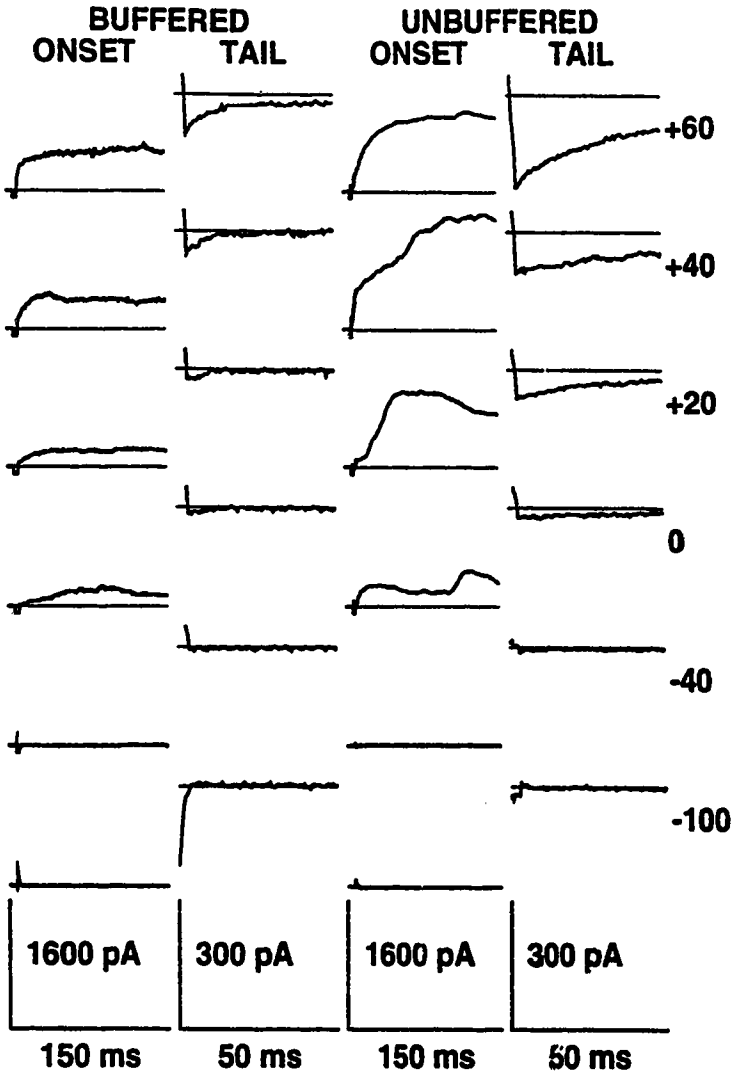

Fig. III-5. Outward currents recorded with the low calcium pipette solution (columns 1 and 2) and the low calcium solution from which EGTA and calcium were omitted (columns 3 and 4). The holding potential was -70 mV. For each row, the command potential appears on the extreme right. The response to these command potentials is shown in columns 1 and 3 (ONSET). The tail currents upon repolarization to -70 mV are shown in columns 2 and 4 (TAIL). The step to the command potential occured 5 ms into the sweeps shown in columns 1 and 3, and repolarization occured 1 ms prior to the sweeps shown in columns 2 and 4.

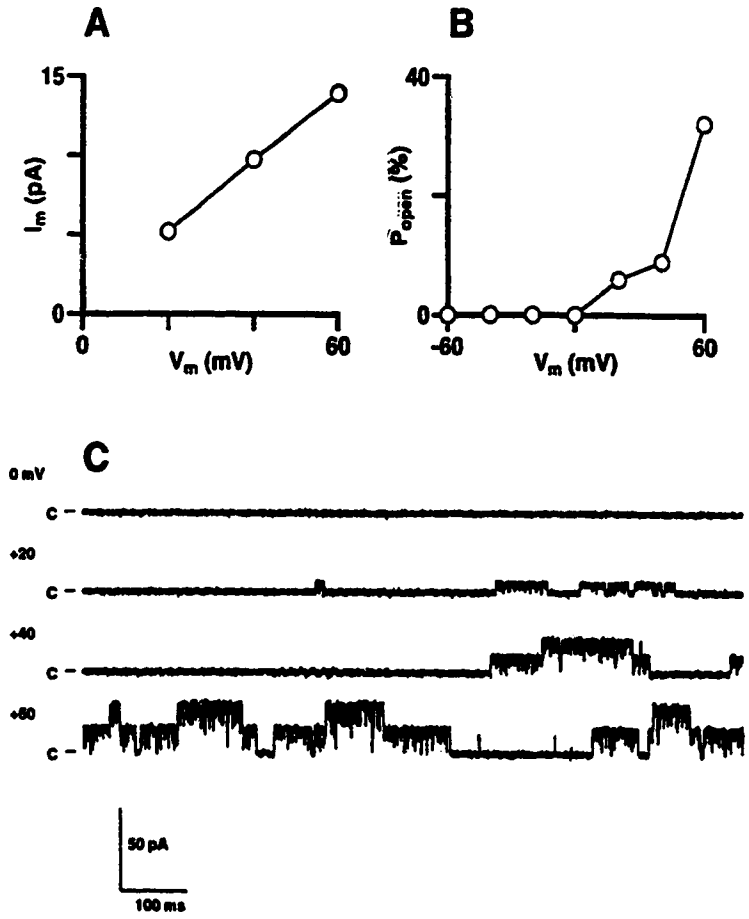

Fig. III- 6. Large conductance calcium-activated potassium channels in cell-free inside-out membrane patches. A. In symmetric 140 mM potassium, the relationship between single channel current and membrane holding potential had a slope of 218 pS. B. Channel open probability was computed as the mean number of channels open in 120 s divided by 2, the number of channels apparently in the patch. C. One second samples of ionic currents are shown from recordings made at holding potentials of 0-60 mV. The closed-state amplitude is marked. As in whole-cell recordings, outward currents are shown as upward deflections.

CHAPTER FOUR

EFFECTS OF PINACIDIL, CROMAKALIM AND NICORANDIL ON POTASSIUM CURRENTS OF RAT BASILAR ARTERY SMOOTH MUSCLE

SUMMARY

Whole-cell and cell-free inside-out patch-clamp recording techniques were used to examine the actions of potassium channel openers (nicorandil, pinacidil and cromakalim) in enzymatically isolated smooth muscle cells of rat basilar artery. Delayed rectifier (K_V) and calcium-activated potassium current (BK_{Ca}) were identified from the whole cell recordings. The amplitude of the K_V was substantially reduced by exposure to procaine (1 mM) and was almost completely eliminated by strychnine (0.5 mM). The BK_{Ca} was not blocked by exposure to 1 mM procaine, but was blocked by TEA (1 mM). Only the BK_{Ca} was increased by a high concentration of cromakalim (10-100 μM) and pinacidil (100-200 μM). BK_{Ca} was decreased after the drugs were washed out. Nicorandil (100-900 μM) affected neither whole-cell current component. Recordings from inside-out membrane patches revealed a large conductance calcium-activated potassium channel, which presumably underlies most of the BK_{Ca} observed in whole-cell recordings. Cromakalim and pinacidil at the same concentration as in whole-cell study, increased the open probability of this channel. Nicorandil produced a dose-dependent increase in half of the experiments. The others demonstrated increases of P_{open} at 100 μM in the first 5 min. Then with increases in the nicorandil concentration, the channel activities either remained unchanged or showed fewer openings than in control solution. On the basis of these results, we suggest that cromakalim and pinacidil only at, high concentration, open calcium-activated potassium channels in rat basilar artery smooth muscle cells. The mechanism of

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nicorandil remained to be identified.

INTRODUCTION

Pinacidil, nicorandil and cromakalim are antihypertensive agents, which relax peripheral vascular smooth muscles by hyperpolarizing smooth muscle cells towards the potassium equilibrium potential (Southerton et al., 1988; Weir and Weston, 1986). These drugs increase $^{86}Rb^+$ or $^{42}K^+$ efflux and reverse or attenuate the effects of a wide variety of vasoconstrictors (Hamilton et al., 1989). Patch-clamping studies show that these drugs act on several types of potassium channels. In cardiac myocytes, nicorandil (Hiraoka and Fan, 1989), pinacidil (Escande et al., 1989) and cromakalim (Sanguinetti et al., 1988) usually activate a background, time- and voltage-independent potassium current which is blocked by physiological levels of ATP. In arterial and venous smooth muscles, these agents activate either large-conductance calcium-activated potassium channels (Gelband et al., 1989) or ATP-dependent potassium channels (Standen et al., 1989). Nicorandil has a dual action, a stimulation of guanylate cyclase and an increase in a potassium conductance (Newgreen et al., 1988).

Some previous studies have been directed at the actions of these potassium channel openers on intact cerebral vessels. Pinacidil has been shown to be less effective at relaxing canine cerebral arteries than coronary, renal and mesenteric arteries in an isometric tension study (Toda et al., 1985), nicorandil was found to hyperpolarize canine cerebral artery and partially reverse canine cerebral vasospasm (Harder et al., 1987) and cromakalim improved rabbit brain blood flow (Hof et al., 1988).

In this study, we used whole-cell and cell-free patch-clamp techniques to assess the potential effects of these drugs in cerebral vasculatures and to determine what types of potassium channels were activated by these agents in enzymatically isolated smooth muscle cells from rat basilar artery. The potential use of these drugs in the treatment of cerebral disorders, especially cerebral vasospasm after subarachnoid hemorrhage, is also discussed.

MATERIALS AND METHODS

Isolation of Rat Basilar Artery Vascular Smooth Muscle Cells

Sprague-Dawley rats were anesthetized with halothane and decapitated. The basilar arteries were removed to a medium consisting of (mM): NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, HEPES 10, penicillin (100 U/ml) and streptomycin (0.1 g/l). Arteries were then cleaned of connective tissues and small side branches. The basilar artery was moved to a medium in which the $CaCl_2$ was reduced to 0.2 mM and to which was added collagenase (Type II, 0.5 g/l), elastase (0.5 g/l), hyaluronidase (Type IV-S, 0.5 g/l), and deoxyribonuclease I (0.1 g/l). The arteries were cut into 0.2 mm rings and incubated for 1 h at room temperature. The rings were transferred to fresh solution containing $CaCl_2$ (0.2 mM), trypsin inhibitor (0.5 g/l) and deoxyribonuclease I (0.1 g/l) and then triturated gently. Cells were plated on glass cover slips and stored at 4°C in saline containing $CaCl_2$ (0.8 mM) and essentially fatty-acid-free bovine serum albumin (2 g/l).

Whole-cell Recording Techniques

Whole cell currents were recorded using standard techniques (Hamill et al., 1981) and an Axopatch 1C patch clamp amplifier (Axon Instruments). The patch pipettes had tip resistances of 1-4 MΩ and seal resistances of over 1 GΩ. Series resistance compensation was not employed. Under whole cell patch clamp conditions, the cells had an input resistance of 5-10 GΩ and a capacitance of 20-30 pF. The membrane potentials and current signals were stored on a laboratory computer. Leakage conductance was determined by applying small, hyperpolarizing voltage steps. None of the records shown was leakage corrected. The normal bath solution for the whole cell recordings was (mM): NaCl 130, KCl 5.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8 (BK_{Ca}) or 0 (K_V), HEPES 10, glucose 5.2 and the pH was adjusted to 7.4 with NaOH. Pipettes were filled with (mM): KCl 139, $MgCl_2$ 0.5, $CaCl_2$ 0.1 (BK_{Ca}) or 0 (K_V), EGTA 0.09 (BK_{Ca}) or EDTA 10 (K_V), HEPES 10, glucose 10 and the pH was adjusted to 7.4 with KOH. Bath solution changes were made with a syringe pump (Sage model 351), modified for simultaneous withdrawal and injection. All experiments

were done at room temperature (19-22°C).

Single Channel Recording Techniques

Single channel current recordings were conducted at room temperature in the inside-out configuration (Hamill et al., 1981). The bath (intracellular) solution had the following composition (mM): KCl 140, $CaCl_2$ 0.008, $MgCl_2$ 0.5, HEPES 10, EGTA 0.09, glucose 10 and the pH was adjusted to 7.4 with KOH. The free calcium concentration in this solution was calculated (Stockbridge, 1987) to be 1.5 nM. The pipette medium (extracellular solution) contained (mM): KCl 140, $CaCl_2$ 0.1, $MgCl_2$ 0.5, HEPES 10, Glucose 10, pH 7.4 with KOH. Single channel recordings were made with the Axopatch 1C amplifier and stored digitally on video tape (Neurocorder model 384 digitizer and Sony SL-700 VCR). On playback, data were redigitized at 10 KHz with a DT-2841-F (Data Translation) analog-digital converter on an AT-compatible computer. Amplitude histograms were made from contiguous segments of data about 5 min long. Channel openings were detected as crossings of 50% or 150% thresholds between histogram peaks.

Drugs

Drugs were pinacidil (Leo), cromakalim (Beecham), nicorandil (Upjohn). Other agents were purchased from Sigma. Stock solutions of cromakalim and pinacidil (10 mM) were made up in 95% ethanol. Nicorandil (10 mM) was dissolved in HCl at pH 3 or 95% ethanol. Control experiments established that the vehicle was not responsible for changes in potassium currents that were observed.

RESULTS

WHOLE-CELL RECORDING

A small inwardly rectifying potassium current and spontaneous transient outward currents were visible in some records. Because of the variable nature of their appearance, the effect of potassium channel openers on these currents was not studied.

Delayed Rectifier

With no added calcium in the pipette and bath solutions, a potassium current (Fig.

IV-1) was obtained. This current activated at about -30 mV and showed outward rectification above +50 mV. This current showed slow sigmoidal activation and no inactivation over the 150 ms time course of the command potential. The amplitude of this current was substantially reduced by exposure to procaine and was almost completely eliminated by strychnine. These characteristics were similar to those of the K_V observed in many other preparations (Hille, 1984) and it will be subsequently referred to by that name. The maximum magnitude of this current was a few hundred picoamperes.

The effects of nicorandil, pinacidil and cromakalim were determined on K_V. In 18 separate experiments, nicorandil (n = 7), pinacidil (n = 7) and cromakalim (n = 4) exerted no effect on the K_V. They modified neither the amplitude nor the steady-state activation curve of this current. The concentrations of nicorandil were increased cumulatively from 100-900 μM; cromakalim and pinacidil were tried from 50-150 μM and 100-250 μM, respectively.

Calcium-activated Potassium Current

With the high calcium pipette and bath solutions, the potassium current was much larger, usually several nanoamperes (Fig. IV-2, control). The current activated beginning about -50 to -30 mV. At any given potential, this current activated more quickly than did the K_V. Like the K_V, this current showed no inactivation in 150 ms. The current was not blocked by exposure to 1 mM procaine, but was blocked by TEA (1 mM). This current, which includes some contribution from the much smaller K_V, is henceforth referred to as BK_{Ca}.

Application of cromakalim and pinacidil to the external solutions increased the magnitude of the outward potassium currents in the high calcium solutions, as shown in Fig. IV-2 (a, b). At 1-10 μM, pinacidil and cromakalim had no detectable effect on potassium currents in four cells. Pinacidil at 200 μM produced an approximately four-fold increase in the current elicited by a voltage step to +60 mV. In five such experiments, all cells showed increased outward potassium currents upon addition of pinacidil to the bath.

In these five cells, a concentration of 100-200 μM produced increases in peak potassium current of 50-400% (+60 mV). In each of these experiments, after washout with the normal bath solution, currents decreased to near control levels. Four cells exposed to cromakalim experienced increases of the potassium current. The mean concentration required to double the potassium current was about 100 μM. A complete reversal of the current amplitude to control levels was obtained in two of four cells upon washout of the drug. In one other experiment, the cell became leaky during the washout and in the fourth experiment, the current did not decrease during washout.

We did not observe significant increases in potassium current produced by nicorandil in 7 experiments (Fig. IV-2c). Outward currents remained the same as control in six cells at 1-900 μM. Only one cell at 100 μM showed a small increase, but the increase was not reversible with washout.

SINGLE CHANNEL RECORDING

In inside-out membrane patches, calcium-activated potassium channels were easily identified by their large conductance (220 pS), high sensitivity to the cytoplasmic calcium concentration and voltage-dependence (Stuenkel, 1989). Fig. IV-3 shows a short segment of a current recording obtained with depolarization. At potentials positive to 0 mV, these channels were recorded as downward deflections. Some other conductances less than 200 pS were also observed, but were not subjected to study.

All such patches contained multiple potassium channels. The probability of channel openings was reduced by using a low free calcium concentration (estimated to be 1.5 nM) and by holding the patch at about +60 mV. The average number of channel openings was expressed as P_x; a value of 1% P_x indicates that an average of 0.01 channels were opened throughout a certain period. In all such recordings, there were still more than one channel per patch. Consequently, without knowing how many channels, these opening percentages could not be converted to single channel opening probability. However, since each patch was used as its own control, we were still able to show that the drugs increased the channel

opening probability.

After 5 min control recordings, the potassium channel openers were added to the bath (intracellular) medium. At each given concentration, channel activities were recorded for 5 min. In seven inside-out patches (Fig. IV-4a, b), pinacidil (n = 4) and cromakalim (n = 3) all increased the channel open-state probability in a dose-dependent manner. These drugs did not alter channel conductance nor its reversal potential. The increase produced by 200 μM pinacidil was from 2-fold to 5-fold. The largest increases of P_x were produced with cromakalim at 250-300 μM: 1.7- to 7.9-fold. At lower concentrations of 50-200 μM, cromakalim exerted no significant effects. Results obtained with nicorandil (Fig. IV-4c; n=8) were more variable. At 50-300 μM, nicorandil produced a dose-dependent, increase of 1.4- to 2.7-fold in 4 patches. Four other patches demonstrated increases of P_x at 100 μM (in the first 5 min). Then with increases in the nicorandil concentration, two patches exhibited no further changes in channel activities and the other two showed fewer openings than in the control solution.

Discussion

In this experiment, the K_V and the BK_{Ca} were identified from the whole cell recording of enzymatically isolated rat basilar artery smooth muscle cells. K_V and BK_{Ca} were separated from one another pharmacologically and by controlling the intracellular calcium concentration. The K_V current, recorded in a bath containing no added calcium and with a pipette containing EDTA and no added calcium, was typically a few hundred picoamperes in maximum amplitude. Procaine and strychnine were effective blockers of this current. With 1.8 mM calcium in the bath and 90 μM EGTA + 100 μM calcium in the pipette, BK_{Ca} of up to several nanoamperes could be elicited. This current was insensitive to procaine and strychnine, but was blocked by TEA in the bath. All recordings obtained with cell-free inside-out membrane patches contained multiple potassium channels with a conductance of about 220 pS in symmetrical potassium. These typical maxi-K channels (Stuenkel, 1989) probably underlie most of the BK_{Ca} detected in whole cell recordings.

Table IV-1 summarizes voltage-clamp data which relate the action of potassium channel agonists to specific potassium channels. In cardiac muscles, cromakalim (Escande et al., 1988; Osterrieder, 1988; Saguinetti et al., 1988), nicorandil (Hiraoka and Fan, 1989) and pinacidil (Arena and Kass, 1989) all activate a potassium conductance which is non-inactivating and voltage-independent, except for some outward rectification with large depolarizations. This current is now known to be mediated by channels which are blocked at physiological levels of intracellular ATP or other nucleotide triphosphates (Kakei et al., 1985). At 10 μM to a few hundred μM, cromakalim (Escande et al., 1988), nicorandil (Hiraoka and Fan, 1989) and pinacidil (Arena and Kass, 1989; Escande et al., 1989) increase this current by increasing the single channel open probability. One study (Kakei et al., 1986) demonstrated an effect of nicorandil (100-2000 μM) on a time- and voltage-independent potassium channel other than the ATP-dependent potassium channel in cardiac myocytes.

In vascular smooth muscle, these drugs, at concentrations from 0.01 to 10 μM, exert their effects on either the calcium-activated potassium channels or the ATP-dependent potassium channels. In the rabbit aorta, cromakalim (Gelband et al., 1989; Kusano et al., 1987) and pinacidil (Economos et al., 1990; Gelband et al., 1990) increase the macroscopic potassium conductance or the opening probability of single large conductance calcium-activated potassium channels. Similar results have been obtained with cromakalim on vascular smooth muscle cells obtained from the human mesenteric artery (Klöckner et al., 1989) and rabbit portal vein (Hu et al., 1990). Pinacidil has similar effects on smooth muscle cells from the rat azygos vein (Hermsmeyer, 1988). There are other reports of cromakalim and pinacidil activation of another potassium current in vascular smooth muscle. In the rabbit portal vein (Beech and Bolton, 1989), cromakalim activated a potassium current for which the identity is unclear. In another study of single channel recordings (Standen et al., 1989) from the rabbit mesenteric artery, cromakalim clearly activated ATP-dependent potassium channels.

In our experiments, neither the pipette solution used in whole cell recordings nor the bath solution used in recordings from inside-out membrane patches contained nucleotide triphosphates. Since time-and voltage-independent current obtained under these conditions was small, we did not try to identify whether there is a ATP-dependent potassium channel by changing the concentration of cytoplasmic ATP or using any specific antagonists of ATP-dependent potassium channels. There is evidence for such a conductance in some vascular smooth muscles (Standen et al., 1989), but some of this evidence depends upon the use of glibenclamide. Gelband et al. (1990) has shown that glibenclamide is not a specific blocker of ATP-dependent potassium channels, but also blocks calcium-activated potassium channels in rabbit aorta smooth muscle.

We showed that these potassium channel openers have no effects on the K_V from enzymatically isolated smooth muscle cells of rat basilar artery. At concentrations above those required to produce increases in the BK_{Ca}, no effect on the amplitude, time course or voltage-sensitivity of the K_V was found. The results from this study of whole-cell and single channel recordings suggested that large conductance calcium-activated potassium channels were activated by a high concentration of pinacidil and cromakalim in rat basilar artery. In whole-cell studies, outward currents decreased to control levels after the drugs were washed out, showing that the effects of pinacidil and cromakalim were reversible. Recordings from cell-free membrane patches confirmed that the effects of these drugs on the calcium-activated potassium channels were direct and did not require changes in the intracellular calcium concentration. Pinacidil and cromakalim at higher concentrations showed increases of channel opening percentages, which is consistent with the above mentioned reports using lower concentrations in other species or tissues (Gelband et al., 1989, 1990; Kusano et al., 1987; Economos et al., 1990).

The situation with nicorandil is evidently more complex. We know of no study which has concluded that nicorandil activated a calcium-activated potassium channel. Hiraoka and Fan (1989) suggested that nicorandil exerted its actions on ATP-dependent

potassium channels in rabbit ventricular myocytes and ruled out the possible involvement of K_V and BK_{Ca}. They found that nicorandil mildly depressed the BK_{Ca} by an average of 16%. They also found that nicorandil's effects did not last long and that spontaneous recovery to a level about 20 to 40% of the peak level occurred after 2 to 5 min, despite the continued presence of nicorandil. In our whole cell recordings, nicorandil, at concentrations from 1 to 900 μM, did not significantly increase the outward currents. In our single channel recordings, nicorandil increased the open probability only during the first 5 minutes. At higher concentrations (and later times), channel activities varied considerably. This may represent the time-dependent effects of nicorandil noted by Hiraoka and Fan (1989). A lack of effect on whole-cell currents is consistent with a previous report that nicorandil (100 μM) had no effect on the membrane potential or input resistance of smooth muscle cells studied in the intact guinea-pig basilar artery (Fujiwara and Kuriyama, 1983).

There is evidence that cerebral vasospasm following subarachnoid hemorrhage is accompanied by depolarization of the arterial smooth muscle cells (Harder et al., 1987). This depolarization would be expected to open voltage-dependent calcium channels and thereby raise the intracellular calcium concentration. An intracellular calcium antagonist, HA 1077, blocks development of vasospasm in a canine subarachnoid hemorrhage model (Shibuya et la., 1988). It is reasonable to expect that drugs which prevent the development of smooth muscle cell depolarization (Ahnfelt-Rønne, 1988) would also be effective in the treatment of vasospasm. Potassium channel agonists were suggested as prime candidates for such drugs (Harder et al., 1987). Our results from rat basilar artery smooth muscle cells suggest that pinacidil and cromakalim only at high concentrations activated calcium-activated potassium channels, which may result in cell membrane hyperpolarization. Since cromakalim was reported to be more or equally effective in the basilar artery than in the thoracic aorta of the rabbit (Grant and O'Hara, 1989), and since much higher concentrations of these drugs were needed to open calcium-activated potassium channels in the rat basilar artery than in peripheral arteries (Table 1), the search for additional

mechanisms deserves further investigation.

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Table IV-1. Summary of voltage-clamp data on potassium channel openers in cardiovascular tissue.

Tissue	Drug	[M]	ATP	Ca^{2+}	Other	Tech	Ref
Cardiac Myocytes							
Guinea-pig ventricle	C	100			+	WC	Osterrieder, 1988
	C	30-300	+			WC,SC	Escande, 1988
	P	10	+			WC	Arena, 1989
	P	10-500	+			WC,SC	Escande, 1989
Rabbit ventricle	N	10-300	+	-		WC,SC	Hiroaka, 1989
Guinea-pig papillary m.	C	10	+			WC	Sanguinetti, 1988
Aorta smooth muscle							
Rabbit, bovine	C	0.1-1		+		WC,SC	Kusano, 1987
Rabbit	C	0.05-10		+		SC	Gelband, 1989,1990
	C	?		+		WC	Economos, 1990
	P	0.1-10		+		SC	Gelband, 1990
	P	0.01-1		+		WC	Economos, 1990
Arterial smooth muscle							
Human mesenteric	C	1		+		SC	Klockner, 1989
Rabbit, rat mesenteric	C	1	+			SC	Standen, 1989
Venous smooth muscle							
Rabbit portal	C	10		-	+	WC	Beech, 1989
	C	1		+		WC,SC	Hu, 1990
Rat portal	N	30-500		+		WC,SC	Kajioka, 1990
Rat azygous	P	10		+		WC,SC	Hermsmeyer, 1988

The drugs were C = cromakalim, P = pinacidil and N = nicorandil. The potassium currents associated with the drugs were ATP = time- and voltage-independent potassium current inhibited by ATP, Ca^{2+} = large conductance calcium-activated potassium current and other = unidentified potassium currents. A"+" indicates that the drug acted as an agonist. A "-" indicates that the drug acted as an antagonist. The techniques were WC = whole-cell patch-clamp and SC = single channel.

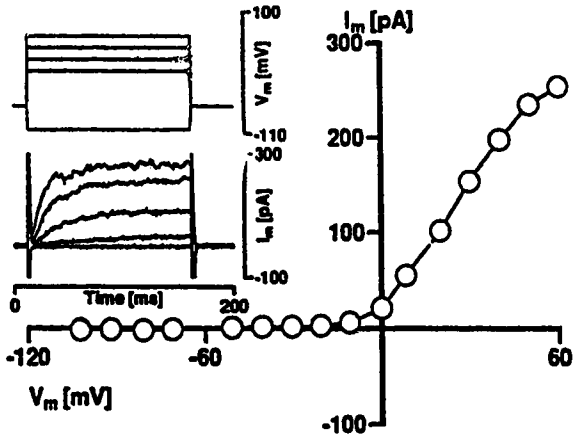

Fig. IV-1. I_K from whole-cell patch-clamp experiments of enzymatically isolated rat basilar artery smooth muscle cells. The inset shows voltage commands (upper traces) and current records (bottom traces). The steady-state current-voltage relationship shows a voltage- and time-dependent potassium current (O). No inward current or inward rectifier current is evident in these records.

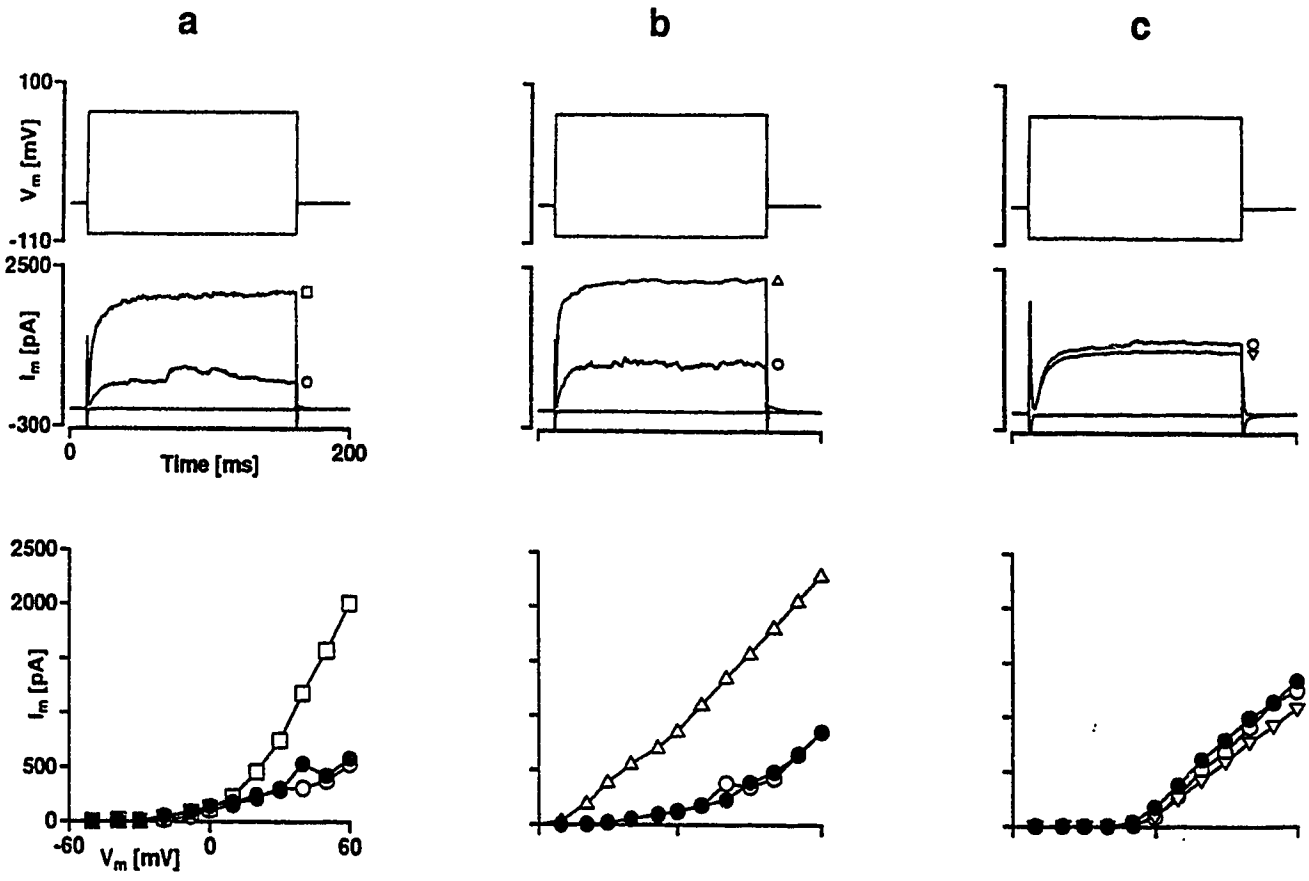

Fig. IV-2. Effects of pinacidil, cromakalim and nicorandil on BK_{Ca} from whole-cell recordings. The upper part of the figure show voltage steps (upper traces) and current records (bottom traces). Current-voltage plots show BK_{Ca} measured in the normal bath solution (O), in the presence of 200 μM pinacidil (a; □), 100 μM cromakalim (b; △) or 400 μM nicorandil (c; ▽), and after wash out (●). Each figure shows results from one cell. Pinacidil and cromakalim reversibly increased BK_{Ca}. Nicorandil had no effect.

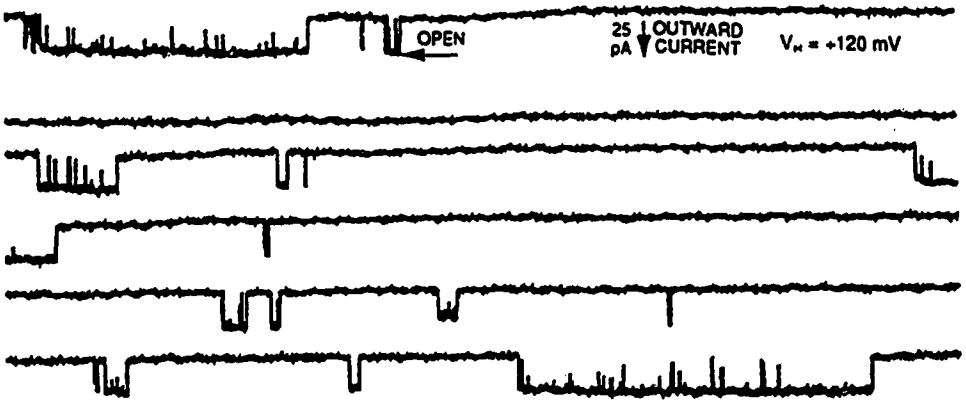

Fig. IV-3. Large conductance calcium-activated potassium channels from cell-free inside-out membrane patches in symmetrical 140 mM potassium. Bath free calcium concentration was estimated to be 1.5 nM. Downward deflections are channel openings. The membrane was voltage-clamped to +120 mV. The channel had a conductance of about 220 pS. Some openings were less than the full height; these probably reflect subconductance levels of the calcium-activated potassium channel.

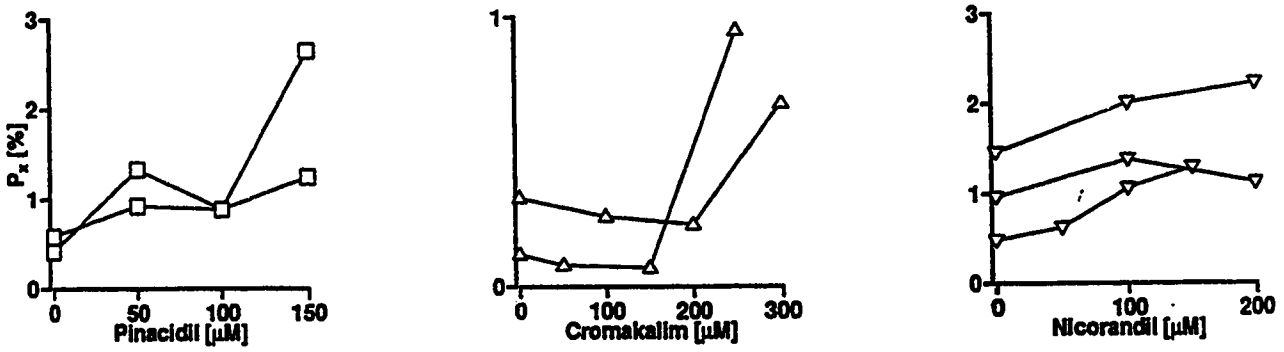

Fig. IV-4. Dose-dependent effects of pinacidil, cromakalim and nicorandil on calcium-activated potassium channel opening percentages from cell-free inside-out membrane patches in symmetrical 140 mM potassium. Bath free calcium concentration was estimated to be 1.5 nM. The membrane was voltage-clamped to +60 mV. The concentration of pinacidil, cromakalim and nicorandil is shown on the abscissa; the channel opening percentage P_x is shown on the ordinate. Each curve represents data from one patch. Cromakalim and pinacidil produced dose-dependent increases in the channel opening percentages. Effects obtained with nicorandil were variable.

CHAPTER FIVE

VASODILATATION OF CANINE CEREBRAL ARTERIES BY NICORANDIL, PINACIDIL AND LEMAKALIM

SUMMARY

Nicorandil, pinacidil and lemakalim relaxed precontracted rings of canine cerebral artery. The order of potency was lemakalim > nicorandil ≅ pinacidil, but all these agents were less effective than nimodipine. The effects of nicorandil were inhibited by methylene blue but not by glibenclamide, while the effects of pinacidil and lemakalim were inhibited by glibenclamide but not by methylene blue. Thus nicorandil probably causes relaxation mostly by effects on guanylate cyclase while lemakalim and pinacidil produce the same effect by action at ATP-dependent potassium channels.

INTRODUCTION

Vasorelaxants nicorandil, pinacidil and cromakalim hyperpolarize smooth muscle cells towards the potassium equilibrium potential (Furukawa et al., 1981; Hamilton et al., 1986; Southerton et al., 1988). These drugs increase $^{86}Rb^+$ or $^{42}K^+$ efflux and reverse or attenuate the effects of a wide variety of directly-acting and endothelium-dependent vasoconstrictors (Hamilton and Weston, 1989). Whole-cell and single-channel studies show that these drugs act on several types of potassium channels. In cardiac myocytes, these agents usually activate a background, time- and voltage-independent potassium current which is blocked by physiological levels of ATP (Hiraoka and Fan, 1989; Sanguinetti et al., 1988; Arena and Kass, 1989). In peripheral arterial and venous smooth muscle, these drugs activate either a large-conductance calcium-activated potassium channel (Gelband et al., 1989; Hu et al., 1990; Klöckner et al., 1989; Kajioka et al., 1990) or ATP-dependent potassium current (Standen et al., 1989). There have been

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few studies of these agents in cerebral arteries and their mechanism of action in this system is not clear. High concentrations of cromakalim and pinacidil increased the activities of a large-conductance calcium-activated potassium channels in rat basilar artery (Zhang et al., 1991a), but cromakalim opened ATP-dependent potassium channels in canine middle cerebral artery (Masuzawa et al., 1990)

In the present study, we have examined the relaxation caused by nicorandil, pinacidil and lemakalim (BRL 38227), the vasorelaxant (-)-trans enantiomer of cromakalim (Edwards and Weston, 1990), in rings of canine basilar and middle cerebral arteries precontracted with prostaglandin $F_{2\alpha}$, and compared the effects of these drugs with those of nimodipine, an agent which blocks the L-type calcium channel and which has been extensively investigated in canine cerebral arteries (Nosko et al., 1986). The mechanism of these drugs in canine basilar arteries was investigated using the potassium channel antagonist, glibenclamide. This drug blocks the ATP-dependent potassium channels in pancreatic β-cells (Fosset et al., 1988), and probably in cardiac myocytes (Escande et al., 1988; Sanguinetti et al., 1988) and rabbit mesenteric arteries (Standen et al., 1989). In addition, it has been suggested that, at least in the case of nicorandil, the relaxation may also involve effects on the soluble guanylate cyclase of vascular smooth muscle (Holzmann, 1983). We have examined this further by using methylene blue, which is an antagonist of guanylate cyclase (Greutter et al., 1981).

Materials and Methods

Preparation of Arterial Rings

Mongrel dogs of either sex, 14-25 kg in weight, were killed with an intravenous overdose of sodium pentobarbital (60 mg/kg). The brain was rapidly removed. The basilar and middle cerebral arteries were isolated from the brains and quickly immersed in a Krebs' physiological saline solution equilibrated with 95% O_2 and 5% CO_2 at room temperature. The composition of the medium was (mM): NaCl 113.7, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and dextrose 10.1. The arteries were cleaned of connective tissue

and side branches and cut into rings 2 mm long. Arteries were mounted vertically between small hooks in a water-jacketed tissue bath of 10 ml working volume maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). No attempt was made to remove the endothelium.

Arterial Tension Recording

At the beginning of each experiment, the rings were stretched to an initial tension of 500 mg, and allowed to equilibrate for approximately 2 h. During the equilibration period, the bathing medium was changed at 15-20 min intervals to prevent the accumulation of metabolites. Contractions and relaxations were recorded isometrically using strain gauges (model FTO 3, Grass Instrument Co.) connected to a polygraph (model 7D, Grass Instrument Co.). Maximum contractions were obtained with prostaglandin $F_{2\alpha}$ (10 μM) and then the tissues were washed and allowed to recover. After a 1 h recovery, the rings were contracted with prostaglandin $F_{2\alpha}$ (3 μM) to produce approximately 50% of the maximum response. When a stable plateau contraction had been obtained, cumulative doses of the relaxant drugs were administered. The relaxation response to each concentration was allowed to reach a plateau before the next addition was made. At the end of each series of experiments, papaverine in a concentration of 100 μM was applied to produce the maximum relaxation. Experiments in which relaxations were produced with nimodipine were carried out under low-light conditions.

Experiments examining the effects of methylene blue was carried out as follows: basilar arterial rings were precontracted with prostaglandin $F_{2\alpha}$ and dose-response curves for relaxation were established as described above. The arteries were then washed at 10 min intervals over a 90 min period, during which the baseline tension was continuously readjusted. Methylene blue (3 μM) was then added to the bath for 20-30 min. Arteries were again contracted with prostaglandin $F_{2\alpha}$ and the dose-response curve was again obtained in the presence of methylene blue.

A similar protocol was used to assess the effects of glibenclamide (10 μM) on

relaxation by nicorandil, pinacidil and lemakalim. In these experiments, 5-HT was used to produce contraction since glibenclamide inhibits contraction by prostaglandin $F_{2\alpha}$ (Zhang et al., 1991b).

Drugs and Solutions

Drugs used were pinacidil (Leo), nicorandil (Upjohn), lemakalim (Beecham), nimodipine (Miles), glibenclamide (Sigma), papaverine (Sigma), prostaglandin $F_{2\alpha}$ (Sigma) and methylene blue (Fisher). Stock solutions of 10 mM nicorandil, pinacidil, lemakalim and nimodipine were prepared in 95% ethanol. Glibenclamide (10 mM) was dissolved in dimethyl sulfoxide. Control experiments established that effects were not the result of vehicles.

Statistical Analysis

Dose-response results have been expressed as a percentage of the maximum possible relaxation, i.e. the relaxation caused by 100 μM papaverine. The results were expressed as mean ± standard error of the mean and compared using one-tailed t-test. A probability of < 0.05 was considered significant. The pD_2 values and maximum relaxations were calculated by the methods prescribed previously (Zhang et al., 1991b).

RESULTS

The Relaxant Effects of Nicorandil, Pinacidil, Lemakalim and Nimodipine in Canine Basilar and Middle Cerebral Arteries

Nicorandil, pinacidil and lemakalim each produced relaxation in canine basilar and middle cerebral arteries precontracted with prostaglandin $F_{2\alpha}$ (Fig. V-1a,b,c). Nicorandil and pinacidil produced similar relaxation in both basilar and middle cerebral arteries. At 100 μM, nicorandil and pinacidil relaxed canine basilar and middle cerebral arteries close to the maximum relaxation induced by 100 μM papaverine. Lemakalim produced its maximum relaxations at 3-10 μM and although it appeared to produce greater relaxations in middle cerebral artery than in basilar artery, this did not reach statistical significance. The pD_2 values were in the order of lemakalim > nicorandil ≅ pinacidil (Table V-1).

The relaxant effects of nicorandil, pinacidil and lemakalim in canine cerebral arteries were compared with nimodipine. Fig. V-1d shows that canine cerebral arteries are more sensitive to nimodipine than to any of the potassium channel openers. The pD_2 values for nimodipine were 6.6 for basilar artery and 7.7 for middle cerebral arteries; these values are not significantly different.

The Relaxant Effects of Nicorandil, Pinacidil and Lemakalim in Canine Basilar Artery in the Presence of Methylene Blue

Methylene blue (3 μM) slightly enhanced the resting tension and the arterial responses to prostaglandin $F_{2\alpha}$ (data not shown), but had no effects on the relaxations produced by lemakalim or pinacidil (Fig. V-2b, c; Table V-1). Methylene blue appeared to inhibit comparatively the relaxation to nicorandil (Fig. V-2a).

The Relaxant Effects of Nicorandil, Pinacidil and Lemakalim in Canine Basilar Artery in the Presence of Glibenclamide

Glibenclamide reduced resting tension but did not inhibit the contractile responses of canine basilar artery to 5-HT (0.3 μM). At 10 μM, glibenclamide failed to inhibit relaxation induced by nicorandil (Fig. V-3a), but did inhibit the effects of lemakalim (Fig. V-3c) and, to a less degrees pinacidil (Fig IV-3b) (Table V-1).

DISCUSSION

The effects of nicorandil, pinacidil and cromakalim have been extensively studied in vascular smooth muscle in different animal and tissue preparations (Hamilton and Weston, 1989). The results showed that these drugs hyperpolarized the smooth muscle, driving the membrane potential further away from the threshold of voltage-dependent calcium channels, resulting in muscle relaxation (Weston, 1988). However, there have been few studies of these drugs in cerebral arteries. Since cerebral arteries may behave differently from peripheral arteries (Toda, 1977), it is important to know whether these drugs work the same way in cerebral arteries as they do in peripheral arteries, and by what mechanisms. Since prostaglandins were rarely used as agonists in most of the studies of

potassium channel openers, and since prostaglandins play an important role in the control of cerebral circulation (Wahl, 1989a), prostaglandin $F_{2\alpha}$ was employed as the agonist in the first part of this study.

The effects of nicorandil, pinacidil and lemakalim in canine cerebral arteries were assessed using isometric tension recordings. All of these drugs significantly relaxed the contractions induced by prostaglandin $F_{2\alpha}$. In this study there were no significant differences between the basilar and middle cerebral arteries in the relaxant effects of nicorandil and pinacidil. Lemakalim appeared to achieve half its peak effectiveness at lower dose than pinacidil and nicorandil in both basilar and middle cerebral arteries, but this difference is not statistically significant.

Since some calcium channel blockers were clinically used against vasospasm after subarachnoid hemorrhage, the effects of potassium channel openers in cerebral arteries have been compared with them in some studies. The actions of pinacidil was found more pronounced in canine peripheral arteries than in cerebral arteries, while nifedipine, a calcium channel blocker, relaxed cerebral arteries better than peripheral arteries (Toda et al., 1985). In contrast, the effects of pinacidil in feline pial arteries of the parietal cortex is about 100 times greater than that of the calcium entry blocker nimodipine (Wahl, 1989b). Cain and Nicholson (1989) compared the effects of cromakalim and nimodipine in rings from rabbit basilar arteries. 5-HT induced two components of contraction in basilar artery, and only the first component was cromakalim sensitive, while nimodipine depressed both components of the contraction. The effects of potassium channel openers in this study in canine cerebral arteries were compared with those of nimodipine, a dihydropyridine calcium channel antagonist. Nimodipine at lower concentrations produced more relaxation of prostaglandin $F_{2\alpha}$-induced contractions than any of these potassium channel openers.

In the present study, the effects of nicorandil, but not pinacidil and lemakalim, were blocked by methylene blue, a inhibitor of guanylate cyclase (Greutter et al., 1981). It is likely that the effects of nicorandil in canine cerebral arteries are mainly mediated by its

activation of guanylate cyclase, as its effects were attenuated by methylene blue. In examining the effects of these agents on ATP-dependent potassium channels, glibenclamide was used as the antagonist. Prostaglandin $F_{2\alpha}$ is not an appropriate agonist for these studies, since glibenclamide is believed to have a direct effect on the action of prostaglandin $F_{2\alpha}$ in canine cerebral arteries (Zhang et al., 1991b). Thus 5-HT was used to induce the contraction for this study. Glibenclamide was without significant effects on the relaxation induced by nicorandil, and it thus seems improbable that nicorandil owes its action in cerebrovascular smooth muscle to effects on ATP-dependent potassium channels. On the other hand glibenclamide but not methylene blue significant inhibited the effects of lemakalim, and this suggests that, in contrast to nicorandil, lemakalim promotes relaxation not through effects on guanylate cyclase, but from its known action on potassium conductance (Hamilton and Weston, 1989; Standen et al., 1989). A recent study (Masuzawa et al., 1990) reported that cromakalim relaxed the contraction of canine middle cerebral artery induced by low KCl (20 mM) and this effect was antagonized by glibenclamide. However cromakalim also activates calcium-activated potassium channels in vascular smooth muscles (Gelband et al., 1989; Zhang et al., 1991a; Hu et al., 1990) and glibenclamide also inhibits calcium-activated potassium channels in vascular smooth muscles (Gelband et al., 1989; Hu et al., 1990; Tseng et al., 1990). Therefore, we could not exclude the possibility that cromakalim (or lemakalim) may open two types of potassium channels in cerebral arteries or open one type of potassium channel which is regulated both by intracellular ATP and calcium concentrations. Since the effects of pinacidil were not affected by methylene blue but were somewhat inhibited by glibenclamide and the effects of pinacidil are reported to be sensitive to tolbutamide, another ATP-dependent potassium channel blocker, in feline pial arteries in situ (Wahl, 1989b), we believe that the effects of pinacidil are at least partly mediated by opening potassium channels in cerebral arteries. Our results are substantially in agreement with a recent study using canine coronary arterial smooth muscle (Yanagisawa et al., 1990). Glibenclamide abolished the relaxant effect of

cromakalim, but only slightly attenuated the relaxant effects of pinacidil and nicorandil. They believe that cromakalim is a more specific potassium channel opener than pinacidil and nicorandil.

Although the effects of these potassium channel openers in cerebral arteries were studied in different animals and tissues, by different methods, with different agonists, and with different results, nicorandil (Harder et al., 1987), pinacidil (Wahl, 1989b) and cromakalim (Cain and Nicholson, 1989) have all been suggested as being potentially useful in the treatment of cerebral vasospasm after subarachnoid hemorrhage by hyperpolarizing the smooth muscle membrane. An *in vivo* study that showed that nicorandil partly reversed experimental cerebral vasospasm in the canine basilar artery (Harder et al., 1987). Whether any of these potassium channel openers could play a role in the treatment of cerebral diseases, especially cerebral vasospasm after subarachnoid hemorrhage deserves further study.

In summary, in this study the effects of the three potassium channel openers, lemakalim, nicorandil and pinacidil were investigated in ring preparations from canine cerebral arteries. All agents produced a relaxation of arteries which had been precontracted with prostaglandin $F_{2\alpha}$ or with 5-HT. In the case of nicorandil the relaxation was significantly attenuated by methylene blue, an agent which interferes with soluble guanylate cyclase, and this supports the idea that nicorandil owes its action to effects which involve this enzyme. The relaxations produced by lemakalim or pinacidil were not affected by methylene blue. Glibenclamide, a drug which blocks ATP-dependent potassium channels, was also used to help to clarify the mechanism of action of these agents. Glibenclamide antagonized the relaxation produced by lemakalim and, to a lesser extent, the relaxation produced by pinacidil. It was, however, devoid of significant effects on the relaxation produced by nicorandil, and it thus seems reasonable to suppose that these agents work by different mechanisms, lemakalim and pinacidil causing their effects by action on potassium conductance and nicorandil by action on soluble guanylate cyclase.

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Table V-1. The maximum relaxations and pD_2 values of nicorandil, pinacidil and lemakalim in canine basilar and middle cerebral arteries.

	Nicorandil			Pinacidil			Lemakalim		
	n	R_{max}	pD_2	n	R_{max}	pD_2	n	R_{max}	pD_2
BA	9	77	5.8	12	90	5.4	12	61	6.1
MCA	5	80	5.4	4	93	5.4	6	75	6.4
BA									
Methy-	8	89	5.5	5	93	5.1	6	52	6.0
Methy+	8	90	4.0	5	99	5.0	6	46	6.1
BA									
Gliben-	4	80	5.8	4	99	5.2	4	73	6.0
Gliben+	4	80	5.8	4	99	4.8	4	57	4.3

BA = basilar artery, MCA = middle cerebral artery, Methy- = without methylene blue, Methy+ = with methylene blue, Gliben- = without glibenclamide, Gliben+ = with glibenclamide, R_{max} = maximum relaxation (the relaxation by 100 μM papaverine as 100%), $pD_2 = -\log ED_{50}$.

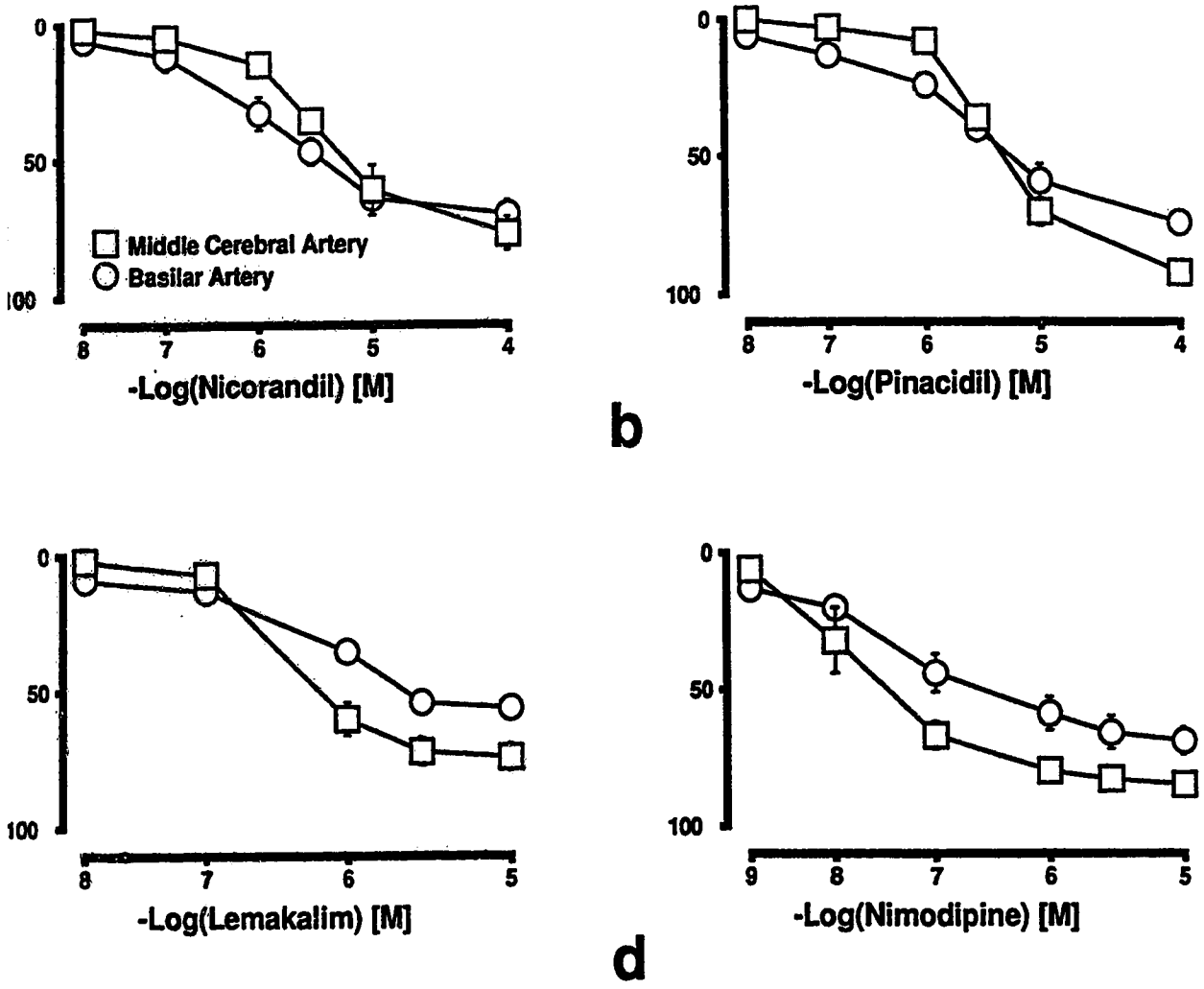
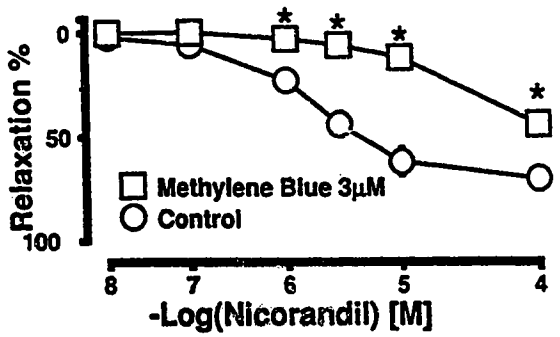
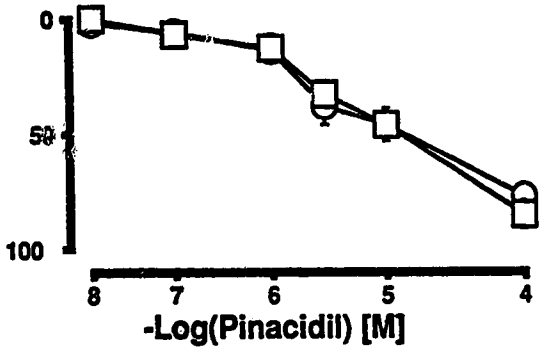


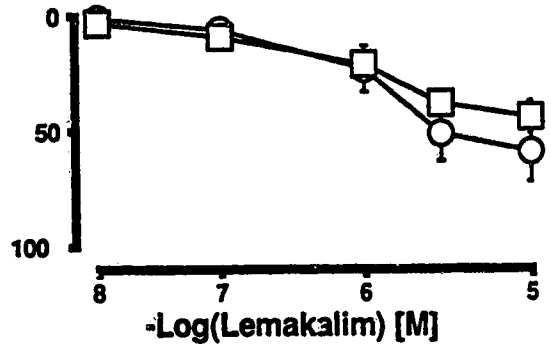
Figure V-1. Relaxant effects of nicorandil (a, n = 9, 5, for basilar and middle cerebral arteries respectively), pinacidil (b, n = 12, 4), lemakalim (c, n = 12, 6) and nimodipine (d, n = 4, 6) in canine basilar (circle) and middle cerebral arteries (square) precontracted by prostaglandin $F_{2\alpha}$ (3 μM). Error bars indicate one standard error of the mean. Relaxation was expressed as percentage of the relaxation by papaverine (100 μM).



a

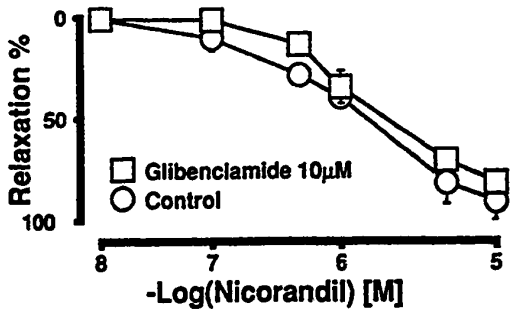


b

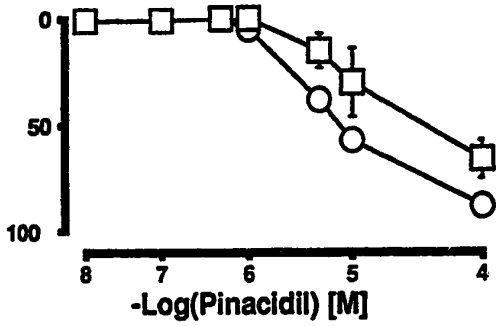


c

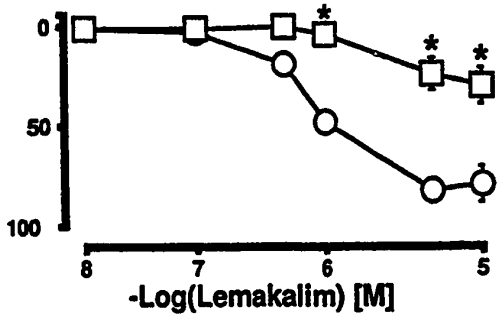
Figure V-2. Relaxant effects of nicorandil (a, n = 8), pinacidil (b, n = 5) and lemakalim (c, n = 6) in the presence (circle) and absence (square) of methylene blue (3 μM) in canine basilar arteries precontracted by prostaglandin $F_{2\alpha}$ (3 μM). Error bars indicate one standard error of the mean. Relaxation was expressed as percentage of the relaxation by papaverine (100 μM). Asterisks represent the significant differences ($p<0.05$) from control.



a



b



c

Figure V-3. Relaxant effects of nicorandil (a), pinacidil (b) and lemakalim (c) in the absence (circle) and presence (square) of glibenclamide (10 μM) in canine basilar arteries precontracted by 5-HT (0.3 μM). Each data point represent 4 rings. Error bars indicate one standard error of the mean. Relaxation was expressed as percentage of the relaxation by papaverine (100 μM). Asterisks represent the significant differences ($p<0.05$) from control.

CHAPTER SIX

GLIBENCLAMIDE RELAXES VASCULAR SMOOTH MUSCLE CONSTRICTION PRODUCED BY PROSTAGLANDIN $F_{2\alpha}$

SUMMARY

The present study has demonstrated: 1) Glibenclamide can reduce resting tension in canine cerebral arteries but has no effect on resting tension in the rat aorta. 2) Glibenclamide can relax prostaglandin $F_{2\alpha}$-induced contractions in the rat aorta, and in canine femoral, mesenteric, renal, coronary, basilar and middle cerebral arteries. 3) The relaxation produced by glibenclamide in rat aorta is comparable to that of glyceryl trinitrate and stronger than that of papaverine. 4) Canine femoral arteries are less sensitive to glibenclamide than the other arteries. 5) In cerebral arteries glibenclamide was as effective as papaverine, but less effective than glyceryl trinitrate. 6) The actions of glibenclamide on cerebral arteries are not mediated by cGMP as they were not blocked by methylene blue, an inhibitor of guanylate cyclase. 7) The effects of glibenclamide are not endothelium-dependent. The mechanism by which glibenclamide produces relaxation is not clear; while the drug is known to block ATP-dependent potassium channels, in vascular smooth muscle this would cause contraction, not dilation. The action of glibenclamide may be at the level of the receptor or the signal transduction process.

INTRODUCTION

Sulfonylureas are hypoglycemic agents that are widely used in the treatment of diabetes mellitus (Loubatières, 1977). It has been shown that sulfonylureas act principally by decreasing potassium permeability in the β-cell membrane (Ferrer et al., 1984), thus leading to β-cell membrane depolarization. $^{86}Rb^{+}$ efflux techniques and electrophysiological methods have recently demonstrated that sulfonylureas block ATP-

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dependent potassium channels in pancreas (Schmid-Antomarchi et al., 1987; Fosset et al., 1988), cardiac ventricular cells (Sanguinetti et al., 1988; Escande et al., 1989) and some peripheral arteries (Standen et al., 1989). It has also been reported that glibenclamide (glybenclamide, glyburide) can block calcium-activated potassium channels (Gelband et al., 1990). Glibenclamide can block or attenuate the vasorelaxant effects of cromakalim, pinacidil, diazoxide, minoxidil sulfate and RP 49356 (Winquist et al., 1989; Eltze, 1989b).

We have observed that glibenclamide not only blocked the actions of cromakalim on the canine basilar artery, but that at a concentration of 10 μM, this agent could decrease resting tension and inhibit or reverse the constriction produced by prostaglandin $F_{2\alpha}$ (Zhang et al., unpublished). This is in contrast to numerous previous reports that glibenclamide did not decrease resting tension of rat or rabbit aorta, nor relax contractions induced by norepinephrine in rat aorta. The available literature on the effects of glibenclamide on smooth muscle is summarized in table 1. In an attempt to resolve the discrepancy between our findings and those from other laboratories, we have used canine cerebral and peripheral arteries as well as rat aortic rings. The importance of the observation lies in the possibility that glibenclamide could have vasorelaxant properties which might show selectivity for one agent or one vascular bed.

Materials and Methods

Preparation of Arterial Rings

Rats were killed by inhalation of halothane. The aorta was removed and immersed immediately in Krebs physiological saline solution. Mongrel dogs of either sex, 14-20 kg in weight, were killed with an intravenous overdose of sodium pentobarbital (60 mg/kg). The brain, mesenteric, renal, coronary and femoral arteries were rapidly removed. The basilar and middle cerebral arteries were isolated from the brains, and the basilar, middle cerebral and other arteries were quickly immersed in a Krebs' physiological saline solution equilibrated with 95% O_2 and 5% CO_2 at room temperature. The composition of the medium was (mM): NaCl 113.7, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4

1.2 and dextrose 10.1. The arteries were cleaned of connective tissue and side branches and cut into rings 2 mm long (Kanamaru et al., 1987). Arteries were mounted vertically between small hooks in a water-jacketed tissue bath of 10 ml working volume maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). In some cases, the endothelium was removed using a stainless-steel wire (Lee, 1982).

Arterial Tension Recording

At the beginning of each experiment, the rings were stretched to an initial tension of 500 dynes for basilar and middle cerebral arteries or 1000 dynes for the canine femoral, mesenteric, renal and coronary arteries and rat aorta, and allowed to equilibrate for approximately 2 h. During the equilibration period, the bathing medium was changed at 15-20 min intervals to prevent the accumulation of metabolites. Contractions and relaxations were recorded isometrically using strain gauges (model FTO 3, Grass Instrument Co.) connected to a polygraph (model 7D, Grass Instrument Co.). Maximum contractions were obtained with KCl (60 mM) and then the tissues were washed and allowed to recover. The maximum responses of each artery to prostaglandin $F_{2\alpha}$ (10 μM) was determined. After a 1 h recovery, the rings were contracted with of prostaglandin $F_{2\alpha}$ (3 μM) to produce approximately 50% of the maximum response. When a stable plateau contraction had been obtained, cumulative doses of the relaxant drugs were administered. The relaxation response to each concentration was allowed to reach a plateau before the next addition was made. At the end of each series of experiments, papaverine in a concentration of 100 μM was applied to produce the maximum relaxation (Toda, 1974).

Experiments examining the effects of methylene blue were carried out as follows: canine middle cerebral artery rings were precontracted with KCl and prostaglandin $F_{2\alpha}$ and dose-response curves for relaxation were established as described above. The arteries were then washed at 5-10 min intervals over a 60 min period, during which the baseline tension was always readjusted. Methylene blue (10 μM) were then added to the bath for 20-30 min. Arteries were again contracted with prostaglandin $F_{2\alpha}$ and the dose-response curve was

again obtained in the presence of methylene blue.

Drugs and Solutions

Glibenclamide (Sigma), Papaverine (Sigma), glyceryl trinitrate (Nippon Kayaku), prostaglandin $F_{2\alpha}$ (Sigma). Methylene blue (Fisher). Stock solutions of glibenclamide (10 mM) were made up in DMSO, the maximal vehicle concentration were less than 1/1000.

Statistical Analysis

Dose-response results have been expressed as a percentage of the maximum possible relaxation, i.e. the relaxation caused by 100 μM papaverine. The results were expressed as mean ± standard error of the mean and compared using Student's t test. A probability of < 0.05 was considered significant. In the figures, symbols used to denote significance were * ($P < 0.05$), † ($P < 0.01$) and ‡ ($P < 0.001$).

Normalized relaxation curves were fit by a simplex (Nelder and Mead, 1965) method minimizing least-square errors to the empirical function: $R_c = R_{max}(1 - \frac{1}{1 + e^{(c - pD_2)/k}})$

where R_c is the normalized relaxation produced by c, the log (base 10) of the drug concentration, R_{max} is the maximum relaxation produced by the drug, pD_2 is -log of the concentration producing half-maximal relaxation (the ED_{50}), and k is a slope factor. This equation provides an excellent fit to the experimental data and enables an unbiased estimation of R_{max} and ED_{50} parameters to be obtained. When no maximum effect was approached at high concentration, the fit was constrained with an R_{max} value of 100%, as indicated. Because there were few points on the steep part of the dose-response curve, the slope factor was the 'softest' parameter fit.

RESULTS

The Resting Tension of Rat Aorta and Canine Cerebral Arteries

Glibenclamide (10 μM) did not decrease the resting tension of rat aorta, but did decrease the resting tension of canine basilar (data not shown) and middle cerebral arteries (Fig. VI-1). At 3 μM, glibenclamide decreased resting tone of canine basilar or middle

cerebral arteries about 100-150 dynes. At 10 μM, glibenclamide decreased tone by 200-250 dynes (Table VI-1).

The Relaxation of Rat Aorta

Glibenclamide, papaverine and glyceryl trinitrate could relax the contractions induced by 3 μM prostaglandin $F_{2\alpha}$ (Fig VI-2). The relaxant effects (ED_{50} values) caused by each drug on rat aorta were in the order of glyceryl trinitrate > glibenclamide > papaverine (Table VI-2). There were significant differences in relaxation of rat aorta between glyceryl trinitrate, glibenclamide and papaverine. Glyceryl trinitrate produced more relaxation than glibenclamide and papaverine at lower concentrations (10 nM to 1 μM; $p < 0.05$ to 0.001). Glibenclamide cause larger relaxations than papaverine at 10 μM concentrations ($p < 0.05$). Glibenclamide and glyceryl trinitrate produced the same maximal relaxation at 3 to 10 μM.

The Relaxation of Canine Femoral, Mesenteric, Renal and Coronary Arteries

Fig VI-3 shows that glibenclamide produced similar effects on all canine arteries. The ED_{50} values were in the order mesenteric ≡ renal ≡ coronary ≡ middle cerebral > femoral (Table VI-2).

The Relaxation of Canine Cerebral Arteries

Glibenclamide could relax both canine basilar and middle cerebral arteries precontracted by 3 μM prostaglandin $F_{2\alpha}$. The ability of glibenclamide in relaxation of canine basilar artery was comparable to that of papaverine (Fig VI-4). Fig VI-5 shows the dose-response curves of canine middle cerebral artery to glibenclamide, papaverine and glyceryl trinitrate. The relaxations were in the order of glyceryl trinitrate > papaverine ≡ glibenclamide (Table VI-2). Again, glyceryl trinitrate produced more relaxation than papaverine and glibenclamide at lower concentrations (100 nM to 1 μM; $P < 0.05$ to 0.01), while glibenclamide and papaverine shared similar effects ($P > 0.05$).

The Effects in Methylene Blue Pretreated Canine Middle Cerebral Artery

Methylene blue (10 μM) slightly enhanced the resting tone of the canine middle

cerebral artery and increased the responses of artery rings to prostaglandin $F_{2\alpha}$ (data not shown). Fig. VI-6 shows that dose-response curves for glyceryl trinitrate, but not for glibenclamide, were shifted to the right one decade by application of methylene blue. There are significant differences at concentrations of 100 nM and above ($P < 0.05$ to 0.01).

The Effects on Canine Middle Cerebral Arteries With and Without Endothelium

The functional removal of the endothelium was proved by the arterial responses to the calcium ionophore A23187, an endothelium-dependent relaxant (Furchgott and Zawadzki, 1980). Relaxation by A23187 was attenuated by removal of the endothelium of canine middle cerebral arteries (data not shown). The effects of glibenclamide were compared in both endothelium-free or intact arteries (Fig VI-7). The endothelium-independent relaxant, glyceryl trinitrate was also applied. Fig VI-7 shows that removal of endothelium did not attenuate the relaxation produced by glibenclamide or glyceryl trinitrate (Table VI-2).

DISCUSSION

Glibenclamide is a sulfonylurea which is used clinically as an oral hypoglycemic agent (Jackson and Bressler, 1981). The sulfonylureas were derived from the antimicrobial sulfonamide drugs (Melander, 1987) and are believed to bring about release of insulin from pancreatic islet β-cells by blocking ATP-dependent potassium channels in the β-cell membrane (Schmid-Antomarchi et al., 1987; Fosset et al., 1988). The resulting depolarization activates calcium channels and the rise in intracellular calcium leads to release of insulin. Sulfonylureas also block ATP-dependent potassium channels in ventricular myocytes (Escande et al., 1989; Sanguinetti et al., 1988) and smooth muscle cells (Standen et al., 1989).

In the well known UGDP study (1970), it was observed that in patients treated with tolbutamide, which was the most commonly prescribed sulfonylurea, showed a 2.5

times increase in mortality compared with those treated by diet alone. While these data are open to criticism (Williamson and Kilo, 1980), and the role of tolbutamide in cardiovascular mortality is still controversial, the observation did give rise to a great deal of experimental work designed to investigate the effects of the sulfonylureas on heart and blood vessels, in order to explain the initial observation. Several experimental studies have shown inotropic effects of sulfonylureas on the heart in animals (Pogátsa and Dubecz, 1977; Linden and Brooker, 1978), but clinical studies have not shown significant effects in humans (Young et al., 1975; Dubach et al., 1979; Huupponen, 1987).

Intravenous glibornuride or tolbutamide produce no effect on blood pressure in normal subjects (Dubach et al., 1979). Glibenclamide (Deineka et al., 1976) and chlorpropamide (Welch et al., 1986) also have no effect on arterial pressure in rats and diabetic patients. Tolbutamide (Lee et al., 1988), gliclazide and carbutamide do increase blood pressure in the intact dog, in contrast to glibenclamide which reduces it (Pogátsa and Dubecz, 1977). In rings of canine femoral arteries, neither tolbutamide nor its metabolites caused any smooth muscle contraction. Pretreatment with tolbutamide potentiated the contractile response to norepinephrine (Lee et al., 1988). Tolbutamide reversed hypoxic pulmonary vasoconstriction in isolated rat lungs (Robertson et al., 1989).

Variable effects are also reported when the effects of the sulfonylureas are examined for their effects on the development of circulatory problems in diabetics. Prendergast (1984) reviewed the effects of glibenclamide and glipizide on vascular complications in diabetic patients. Platelet aggregation is thought to be involved in the development of those complications. For example, glibenclamide inhibits epinephrine- and collagen-induced platelet aggregation (Klaff et al., 1981). Sulfonylurea treatment is also believed to reverse or prevent diabetic angiopathy (Camerini-Davalos et al., 1983).

There are also relevant effects of glibenclamide on non-vascular systems. Numerous studies show relaxant effects of sulfonylureas on uterine smooth muscle. Tolbutamide and glibenclamide antagonized the contractions evoked by depolarization or

vanadate but did not affect the spontaneous contractions of rat uterus (Villar et al., 1986a, b). Other results suggest that tolbutamide can inhibit contractile effects of oxytocin in the rat uterus (Goldraij et al., 1987).

Sulfonylureas can enhance release of prostaglandin I_2 (El Tahir et al., 1986), guanylate cyclase (Vesely, 1986) and decrease plasma levels of thromboxane B_2 and β-thromboglobin (Brunner et al., 1984; Florkowski et al., 1988), lower plasma prorenin (Luetscher et al., 1989) and stimulate plasminogen activator release (Kuo et al., 1988; Almer, 1984). The enhanced release of prostaglandin I_2 may relate to beneficial effects of sulfonylureas on diabetic angiopathy and platelet aggregation. Sulfonylureas also may reduce serum lipids, especially HDL-cholesterol, by some unknown mechanism (Schrapler et al., 1969). Glibenclamide eliminated hyperpolarization induced by brain ischemia, presumably by blocking neuronal ATP-dependent potassium channels (Mourre et al., 1989).

In the work reported here we have shown that glibenclamide can reduce the resting tension in canine cerebral arteries but not in rat aorta. When tension was induced with prostaglandins $F_{2\alpha}$, glibenclamide caused relaxation of all vascular preparations tested, although cerebral arteries were somewhat more sensitive. While the effects of glibenclamide are a little less profound than those of glyceryl trinitrate, it proved to be as effective as papaverine.

The mechanism by which the relaxation is still in question. The effects of both the nitrovasodilators and EDRF are mediated by actions on guanylate cyclase and are thus inhibited by methylene blue. In these studies the actions of glibenclamide were unaffected by pretreatment with methylene blue. Furthermore, removal of the endothelium by mechanical means did not affect the ability of glibenclamide to produce relaxation. It is thus clear that the actions do not depend on the production of nitric oxide from the endothelium. It is uncertain whether ATP-dependent potassium channels are expressed in cerebrovascular smooth muscle cells (Zhang et al., unpublished), but if they are, it is very

unlikely that they make a substantial contribution to the overall normal potassium conductance. It is thus *a priori* unlikely that glibenclamide is altering the contractility of vascular smooth muscle cells by the same mechanism by which it promotes insulin secretion. Furthermore, if the mechanism did involve blockade of ATP-dependent potassium channels, we would expect that the resulting depolarization would lead to calcium influx through voltage-dependent calcium channels and, hence, to contraction, the opposite response to that observed here. Indeed, the agents like cromakalim that open potassium channels are inhibited by glibenclamide, but themselves produce vascular relaxation.

In rat uterine smooth muscle cells, glibenclamide also produces relaxation. Villar et al. (1986a, b) suggested that this did not arise from effects on potassium conductance but from the ability of the drug to cause an increase in the binding of intracellular calcium. Although this mechanism may apply also to vascular smooth muscle, it fails to explain the observation that glibenclamide can only inhibit the activity of some agonists. The explanation by Goldraij et al. (1987) of the same phenomenon viz: that the contraction of uterine smooth muscle is dependent on prostaglandins, whose production or effects are inhibited by glibenclamide, seems intrinsically more probable.

Table VI-1 lists the reports of effects of sulfonylureas on smooth muscle preparations. Relaxant effects, aside from the present study, have been rarely reported. Since most of the previous reports have dealt with rats, rabbits and guinea-pigs, we considered the possibility that our early results on canine cerebral arteries differed because of species differences. Curtis et al. (1975) suggested that there were species differences in sulfonylureas effects. Tolbutamide exerts positive inotropic effects only in rabbits, cats and rats, whereas human and dog myocardial tissue is unresponsive to the drug. However, we found similar results on agonist-induced tension in the rat aorta and in canine arteries. Our results on resting tension in the rat aorta were consistent with previous reports (Table 1); i.e. glibenclamide had no effect on resting tension in this preparation. Most previous reports

have been based on study of the portal vein, aorta and uterus, so we considered the possibility that our results differed because of the choice of canine cerebral arteries. This hypothesis was not borne out in experiments we performed on canine peripheral arteries and rat aorta. The reduction in agonist-induced tension we observed in rat and canine arteries was similar to effects observed in the rat uterus (Villar et al., 1986a, b; Goldraij et al., 1987) and the rat pulmonary artery (Robertson et al., 1989). Our results about relaxant effects of glibenclamide on the contraction produced by prostaglandin $F_{2\alpha}$ in the rat aorta differ from those of Buckingham et al. (1989), in which norepinephrine was employed as agonist. We considered the possibility that the differences arise because of the use of different agonists. While we have not yet completed a systematic survey of various agonists, we note that glibenclamide has been reported to decrease tension in the rat uterus in the presence of KCl, oxytocin, acetylcholine and vanadate (Villar et al., 1986a, b; Goldraij et al., 1987) and in rat pulmonary artery constricted by hypoxia and diazoxide (Robertson et al., 1989). We have yet to assess effects of other sulfonylureas, but note that tolbutamide relaxes agonist-induced constrictions in rat uterus (Villar et al., 1986a, b; Goldraij et al., 1987) and in rat pulmonary artery (Robertson et al., 1989). A higher concentration is required to relax smooth muscle with tolbutamide.

It thus seems likely that the sulfonylureas are capable of causing relaxation of a variety of vascular preparations, but the diverse properties documented in table 1 suggest that a global effect on some common signal transduction pathway is unlikely to be responsible. It may be that these compounds owe their activity to interference with receptor function or some event which is closely connected with it.

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Table VI-1. Effects of sulfonylureas on smooth muscle preparations.

Preparation	Agonist	Sulfonylurea	μM	ΔTension	Reference
Effect on resting tone or spontaneous activity					
Rat uterus		Glibenclamide	5-80	None	Villar et al., 1986b
Rat uterus		Tolbutamide	250-8000	None	Villar et al., 1986b
Rat uterus		Tolbutamide	100	Decrease	Goldraij et al., 1987
Rat portal vein		Glibenclamide	0.3-3	None	Buckingham et al., 1989
Rat portal vein		Glibenclamide	0.3	Increase	Winquist et al., 1989
Rat portal vein		Glibenclamide	1-10	None	Winquist et al., 1989
Rat portal vein		Glibenclamide	3-10	Decrease	Quast and Cook, 1989
Rat aorta		Glibenclamide	0.1-0.3	None	Quast and Cook, 1989
Rat aorta		Glibenclamide	0.1-3	None	Cavero et al., 1989
Rat aorta		Glibenclamide	0.3-1	None	Newgreen et al., 1989
Rat aorta		Glibenclamide	3-10	None	This paper
Rabbit aorta		Glibenclamide	1-10	None	Quast and Cook, 1989
Rabbit vas def.		Glibenclamide	0.1-1	None	Eltze, 1989b
Guinea-pig pulmonary artery		Glibenclamide	0.1-3	None	Eltze, 1989a
Guinea-pig trachea		Glibenclamide	0.1-10	None	Murray et al., 1989
Guinea-pig taenia-caeci		Glibenclamide	1	None	Perkins et al., 1989
Dog middle cerebral artery		Glibenclamide	3-10	Decrease	This paper
Dog basilar artery		Glibenclamide	3-10	Decrease	This paper
Effect on tension produced by a subsequent exposure to agonist					
Rat uterus	KCl	Glibenclamide	40-80	Decrease	Villar et al., 1986b
Rat uterus	KCl	Tolbutamide	400-4000	Decrease	Villar et al., 1986b
Rat uterus	Oxytocin	Glibenclamide	40	Decrease	Villar et al., 1986a
Rat uterus	ACh	Glibenclamide	80	Decrease	Villar et al., 1986a
Rat uterus	Oxytocin	Tolbutamide	100-1000	Decrease	Villar et al., 1986a
Rat uterus	ACh	Tolbutamide	100-1000	None	Villar et al., 1986a
Rat uterus	Oxytocin	Tolbutamide	100	Decrease	Goldraij et al., 1987
Rat uterus	Oxytocin	Glibenclamide	0.3-10	None	Piper and Hollingworth, 1989
Rat aorta	KCl	Glibenclamide	0.1-3	None	Cavero et al., 1989
Rat aorta	KCl	Glibenclamide	0.3-1	None	Newgreen et al., 1989
Rat pulmonary artery	Hypoxia	Tolbutamide	170-8000	Decrease	Robertson et al., 1989
Rat pulmonary artery	Diazoxide	Tolbutamide	170-8000	Decrease	Robertson et al., 1989
Rabbit aorta	AII	Glibenclamide	10	None	Quast and Cook, 1989
Rabbit aorta	NA	Glibenclamide	3	None	Quast and Cook, 1989
Rabbit renal artery	Caffeine	Glibenclamide	3	None	Wilson and Cooper, 1989
Dog femoral artery	NA	Tolbutamide	100-10000	Increase	Lee et al., 1988
Effect on tension produced by a prior exposure to agonist					
Rat uterus	Vanadate	Glibenclamide	5-80	Decrease	Villar et al., 1986b
Rat uterus	Vanadate	Tolbutamide	31-500	Decrease	Villar et al., 1986b
Rat uterus	KCl	Tolbutamide	250	Decrease	Villar et al., 1986a
Rat uterus	Oxytocin	Tolbutamide	250	Decrease	Villar et al., 1986a
Rat uterus	KCl	Glibenclamide	5	Decrease	Villar et al., 1986a
Rat uterus	Oxytocin	Glibenclamide	5	Decrease	Villar et al., 1986a
Rat aorta	NA	Glibenclamide	1-10	None	Buckingham et al., 1989
Rat aorta	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Rabbit mesenteric artery	NA	Glibenclamide	0.1-10	None	Standen et al., 1989
Dog femoral artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Dog middle cerebral artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Dog basilar artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Dog mesenteric artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Dog renal artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper
Dog coronary artery	$PGF_{2\alpha}$	Glibenclamide	0.1-10	Decrease	This paper

ACh= acetylcholine, AII= angiotensin II, NA= norepinephrine and $PGF_{2\alpha}$= prostaglandin $F_{2\alpha}$.

Table VI-2. Summary of dose-response data obtained with various vasorelaxants.

Fig.	Artery	Drug	R_{max}	pD_2	ED_{50}	k
2	Rat aorta	Gli	95	5.9	1	0.16
		Pap	100[a]	5.2	6	0.65
		GTN	91	6.9	0.1	0.45
3	Dog femoral artery	Gli	100[a]	5.1	8	0.34
	Dog renal artery	Gli	91	5.6	3	0.31
	Dog mesenteric artery	Gli	84	5.6	3	0.26
	Dog middle cerebral artery	Gli	100[a]	5.6	3	0.49
	Dog coronary artery	Gli	93	5.7	2	0.30
5	Dog middle cerebral artery	Gli	100[a]	5.6	3	0.49
		Pap	100[a]	5.7	2	0.39
		GTN	100[a]	6.8	0.2	0.60
6	Dog middle cerebral artery	Gli	100[a]	5.6	3	0.49
		Gli+MetB	100[a]	5.6	3	0.49
		GTN	106	6.7	0.2	0.67
		GTN+MetB	100[a]	5.7	2	0.67
7	Dog middle cerebral artery	Gli	100[a]	5.6	3	0.45
		Gli-Endo	100[a]	5.9	1	0.55
		GTN	95	7.6	0.03	0.38
		GTN+Endo	97	7.9	0.01	0.50

Drugs were Gli=glibenclamide, Pap=papaverine, GTN=glyceryl trinitrate and MetB=methylene blue. -Endo indicates absence of endothelium. Data in each figure were fit to the empirical equation given in the text. R_{max} was the maximum relaxation obtained (%, relative to papaverine); [a] indicates that the fit was constrained with R_{max}=100. pD_2 is -log concentration which produced half-maximal relaxation (for that particular drug) and k is a slope factor. Values for the ED_{50} (μM) were computed from the pD_2 values.

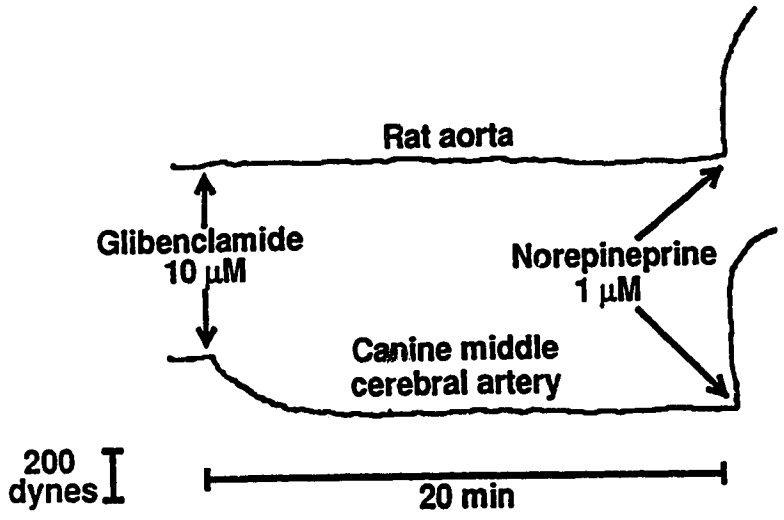


Figure VI-1. Glibenclamide (10 μM) reduced resting tension in the canine middle cerebral artery but not in the rat aorta. Glibenclamide had no effect on the response to a subsequent addition of norepinephrine (1 μM).

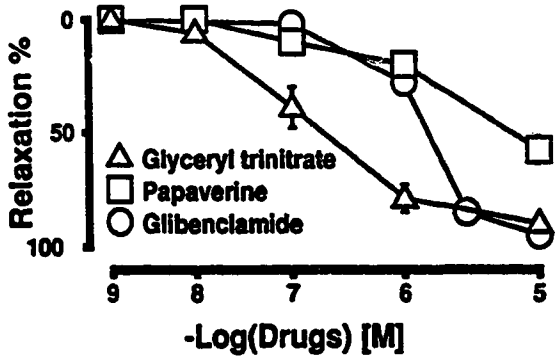


Figure VI-2. Rings of rat aorta were precontracted with prostaglandin $F_{2\alpha}$ (3 μM). The contracted rings were then exposed to either papaverine, glibenclamide or glyceryl trinitrate in serially increasing concentrations from 1 nM to 10 μM. Relaxation parameters, fit with the equation given in text, are shown in Table 2. Bars show the standard errors for a mean of 4 specimens.

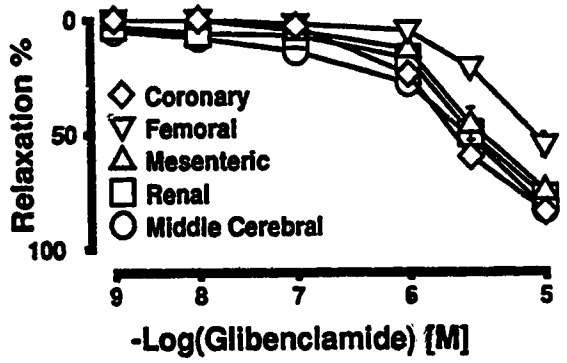


Figure VI-3. Rings of canine femoral, renal, coronary, mesenteric and middle cerebral arteries were precontracted with prostaglandin $F_{2\alpha}$ (3 μM). The contracted rings were then exposed to glibenclamide in serially increasing concentrations from 1 nM to 10 μM. Relaxation parameters, fit with the equation given in text, are shown in Table 2. Bars show the standard errors for a mean of 4 specimens.

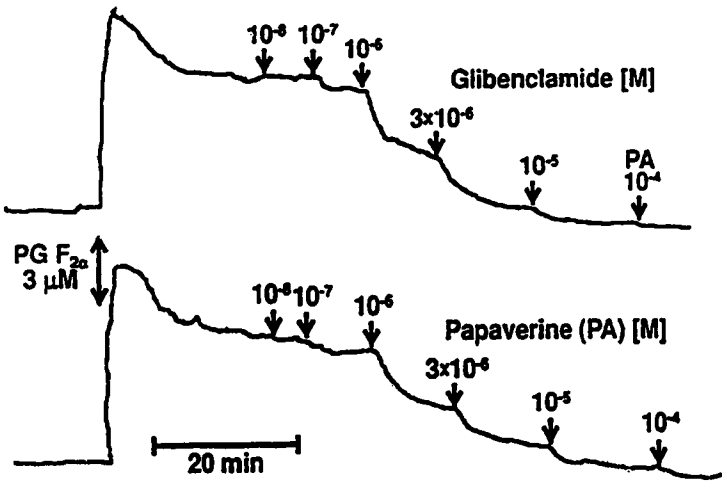


Figure VI-4. Glibenclamide and papaverine had comparable effects on tension in canine basilar artery rings precontracted with prostaglandin $F_{2\alpha}$ (3 μM).

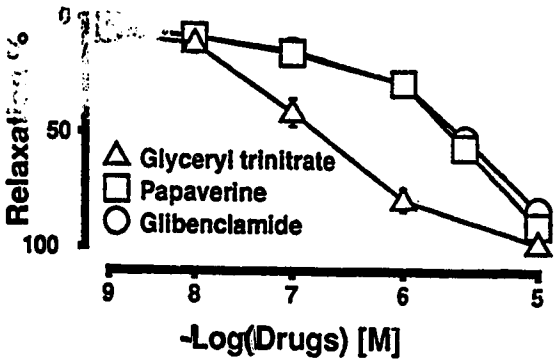
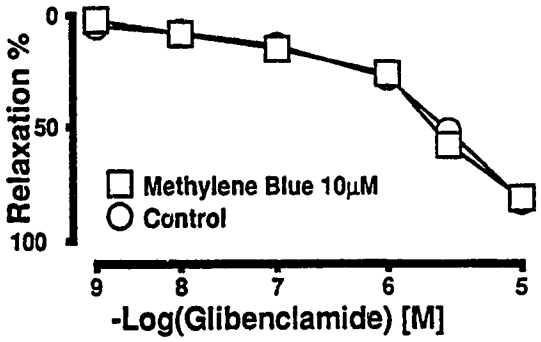
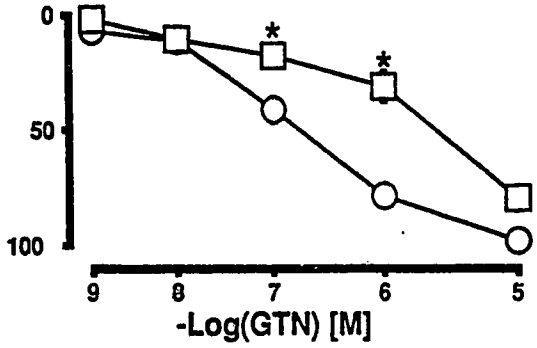


Figure VI-5. Rings of dog middle cerebral artery were precontracted with prostaglandin $F_{2\alpha}$ (3 μM). The contracted rings were then exposed to either glibenclamide, papaverine or glyceryl trinitrate in serially increasing concentrations from 1 nM to 10 μM. Relaxation parameters, fit with the equation given in text, are shown in Table 2. Bars show the standard errors for a mean of 4 specimens.

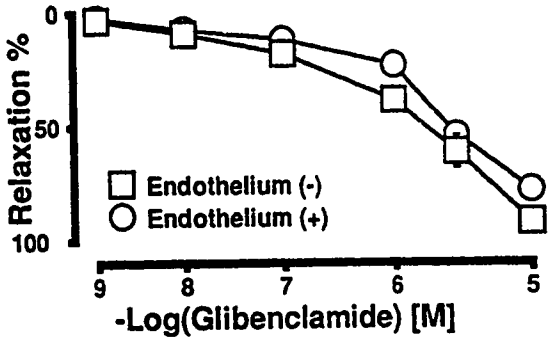


a

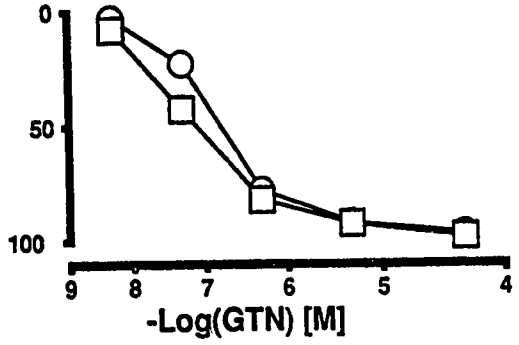


b

Figure VI-6. Rings of dog middle cerebral artery were precontracted with prostaglandin $F_{2\alpha}$ (3 μM) with or without methylene blue (10 μM). The contracted rings were then exposed to either glibenclamide or glyceryl trinitrate in serially increasing concentrations from 1 nM to 10 μM. Relaxation parameters, fit with the equation given in text, are shown in Table 2. Bars show the standard errors for a mean of 4 specimens.



a



b

Figure VI-7. Rings of dog middle cerebral artery, with or without endothelium were precontracted with prostaglandin $F_{2\alpha}$ (3 μM). The contracted rings were then exposed to either glibenclamide or glyceryl trinitrate in serially increasing concentrations from 1 nM to 10 μM. Relaxation parameters, fit with the equation given in text, are shown in Table 2. Bars show the standard errors for a mean of 4 specimens.

CHAPTER SEVEN

GLIBENCLAMIDE INHIBITS THE CONTRACTILE RESPONSES OF CANINE MIDDLE CEREBRAL ARTERY TO EICOSANOIDS AND OXYHEMOGLOBIN

SUMMARY

Glibenclamide is a sulfonylurea used in the management of diabetes mellitus, but which also is known to have antagonist activity against the effects of some eicosanoids on smooth muscle. We have examined the action of glibenclamide against contractions of rings of canine middle cerebral artery produced by prostaglandins $F_{2\alpha}$, E_2 and D_2 and the thromboxane A_2 analog, U46619. All these responses were significantly attenuated by glibenclamide, while contractions to potassium chloride, noradrenaline, 5-Hydroxytryptamine or caffeine were unaffected. The effects of glibenclamide against the vascular actions of oxyhemoglobin were also examined, since this agent is believed to be responsible for the vasospasm which follows subarachnoid hemorrhage. Contractions to oxyhemoglobin were significantly inhibited by glibenclamide, which suggests that at least part of the contractile effects of oxyhemoglobin in cerebral arteries is mediated by eicosanoids. Glibenclamide is thus an agent which selectively blocks the contractile effects of both eicosanoids and oxyhemoglobin.

INTRODUCTION

Glibenclamide (glybenclamide, glyburide) is a sulfonylurea which is used as a hypoglycemic agent for the treatment of non-insulin-dependent diabetes mellitus (Loubatieres, 1977). Its mechanism of action involves blockade of ATP-dependent potassium conductance in pancreatic β-cells which results in depolarization, calcium influx and release of insulin (Schmid-Antomarchi et al., 1987). An unrelated effect of

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glibenclamide was reported recently. Glibenclamide competitively and selectively inhibited the contractile responses of canine coronary artery to a thromboxane A_2 analogue, U46619 (Cocks et al., 1990) and relaxed the contractions produced by prostaglandin $F_{2\alpha}$ in canine cerebral and peripheral arteries and rat aorta (Zhang et al., 1991). Glibenclamide neither inhibited the contractions induced by potassium chloride (KCl), noradrenaline (NA), endothelin-1 (Cocks et al., 1990), angiotensin II (Quast and Cook, 1989) or caffeine (Wilson and Cooper, 1989) in peripheral arteries, nor reversed the relaxation produced by acetylcholine in guinea pig coronary artery (Eckman and Keef, 1991) or cAMP in rabbit colon circular muscle (Willenbucher, 1991).

Cerebral vasospasm is the leading cause of death and disability in patients with aneurysmal subarachnoid hemorrhage (Kassell et al., 1985). Its etiology and pathogenesis are still not well understood, and most of the attempts to prevent or treat this condition have failed (Wilkins, 1986). Oxyhemoglobin is now believed to be the principal cause of cerebral vasospasm (Weir, 1987). There is evidence that oxyhemoglobin may exert its [illegible] by release of eicosanoids (Okamoto et al., 1984). It has been suggested that since eicosanoids may be formed in cerebral vessels as well as brain parenchyma (Wahl et al., 1989) and also can be released from platelets and by stimulation of the brain cortex (Yamamoto et al., 1972), these substances should be considered as factors involved in the regulation of cerebral blood flow and in the pathogenesis of vasospasm. Eicosanoids, such as prostaglandin $F_{2\alpha}$ (Chehrazi et al., 1989), prostaglandin E_2 (Chyatte, 1989), prostaglandin D_2 (Gaetani et al., 1986) and thromboxane A_2 (Komatsu et al., 1986) were increased and prostaglandin I_2 (Nosko et al., 1988) were decreased during the development of vasospasm.

Since some metabolic products from arachidonic acid are associated with the development of cerebral vasospasm and glibenclamide inhibits the contractions by U46619 (Cocks et al., 1990) and prostaglandin $F_{2\alpha}$ (Zhang et al., 1991), it is important to know whether glibenclamide also selectively inhibits other eicosanoids in canine cerebral artery

and whether it can inhibit the contraction produced by oxyhemoglobin. The present study was designed to test 1) the effects of glibenclamide on the contractile responses of canine cerebral arteries to receptor agonists NA, 5-Hydroxytryptamine (5-HT) and prostaglandin $F_{2\alpha}$, and non-receptor agonists KCl and caffeine, 2) the vasorelaxant effects of glibenclamide to the sustained contractions by prostaglandins $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2 and U46619, a thromboxane A_2 analogue, and 3) the inhibitory effects of glibenclamide on the constrictions induced by oxyhemoglobin.

MATERIALS AND METHODS

Preparation of Arterial Rings

Mongrel dogs of either sex, 15-30 kg in weight, were killed with an intravenous overdose of sodium pentobarbital (60 mg/kg). The brains were rapidly removed. The middle cerebral arteries were isolated from the brains, and quickly immersed in Krebs physiological saline solution equilibrated with 95% O_2 and 5% CO_2 at room temperature. The composition of the medium was (mM): NaCl 113.7, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and dextrose 10.1. The arteries were cleaned of connective tissue and side branches and cut into rings 2-3 mm long. No attempt was made to remove the endothelium. Arteries were mounted vertically between small hooks in a water-jacketed tissue bath of 10 ml working volume maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4).

Arterial Tension Recording

At the beginning of each experiment, the rings were stretched to an initial tension of 0.5 g, and allowed to equilibrate for approximately 2 h. During the equilibration period, the bathing medium was changed at 15-20 min intervals to prevent the accumulation of metabolites. Contractions and relaxations were recorded isometrically using strain gauges (Model FTO 3, Grass Instrument Co.) connected to a polygraph (Model 7D, Grass Instrument Co.). Dose response curves for the contractions produced by KCl, caffeine, NA, 5-HT and prostaglandin $F_{2\alpha}$ were obtained first in the absence of glibenclamide, then the

tissues were washed every 15-20 min for 1 hr, and incubated for 15 min with glibenclamide at a concentration of either 1 μM or 10 μM. Further dose-response curves to these agonists were then recorded.

In other experiments, contractions were elicited with prostaglandin $F_{2\alpha}$ (3 μM), prostaglandin D_2 (3 μM), prostaglandin E_2 (3 μM), and U46619 (1 nM); these concentrations produce approximately 50% of the maximum response. When a stable response had been obtained, cumulative doses of glibenclamide were administered, allowing each response to stabilize before the next addition was made. At the end of each series of experiments, papaverine (100 μM) was applied to produce the maximum relaxation.

In the experiments with oxyhemoglobin, the effect of glibenclamide was tested on both the phasic and tonic contractions induced by oxyhemoglobin. In some experiments, oxyhemoglobin (10 μM) was added into the organ bath to produce contraction. When a stable response had been obtained, glibenclamide (0.1 - 10 μM) was administered. The relaxation was expressed as percentage of the stable contraction to 10 μM oxyhemoglobin. In the other experiments, arteries were first incubated for 20 min in glibenclamide (10 μM), then oxyhemoglobin was applied to produce contraction.

Drugs and Solutions

U46619 was a gift of The Upjohn Company. All other agents were purchased from Sigma. Glibenclamide was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the media was less than 0.2%; this concentration was devoid of biological activity. Oxyhemoglobin was produced by reduction of human hemoglobin by sodium dithionite (J.T. Baker Chemical Co.,) (Martin et al., 1985).

Statistical Analysis

Each response was expressed as the mean ± s.e.mean and results were compared using analysis of variance. A probability value of less than 0.05 was considered significant. The pD_2 and maximum contraction values were estimated using the procedure described

previously (Zhang et al., 1991).

RESULTS

1) Glibenclamide reduced the resting tension of canine middle cerebral artery; at concentrations of 1 μM and 10 μM, the resting tension was reduced by 87 ± 14 mg and 185 ± 36 mg respectively (n = 8).

2) Glibenclamide inhibited the contractile responses of canine middle cerebral artery to prostaglandin $F_{2\alpha}$, but had no significant effect on responses to KCl, caffeine, NA or 5-HT. Cumulative application of prostaglandin $F_{2\alpha}$ (Fig. VII-1a), NA (Fig. VII-1b), 5-HT (Fig. VII-1c) and KCl (Fig. VII-1d) produced a vasoconstriction which was concentration-dependent. Pretreatment with glibenclamide (1 or 10 μM) was without significant effect on either the pD_2 values or the maximum responses to KCl, NA or 5-HT (Table VII-1). The responses to lower concentrations of 5-HT showed a small and insignificant reduction in the presence of glibenclamide. In contrast, there was a significant dose-dependent inhibition of the response to prostaglandin $F_{2\alpha}$ ($p<0.05$). The dose-response curves were shifted to the right by glibenclamide. In groups treated with 1 and 10 μM glibenclamide, the maximum contractions were only achieved at agonist concentrations of 100 μM. Caffeine (10 mM) induced a transient contraction in canine middle cerebral artery (n = 4) which was not altered by pretreatment with glibenclamide ($p>0.05$).

3) Glibenclamide relaxed the sustained contractions induced by prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2 and U46619 in canine middle cerebral artery. As shown in Fig. VII-2, glibenclamide caused a similar relaxation on the sustained contractions induced by prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2 and U46619. The pD_2 values and maximum relaxations for glibenclamide to the contractions by prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2 and U46619 do not differ significantly from each other (pD_2 values 5.5 -5.6).

4) Glibenclamide relaxed the contractions induced by oxyhemoglobin. Fig. VII-3

shows the contraction of canine middle cerebral artery by oxyhemoglobin (10 μM). The average contraction by oxyhemoglobin in canine middle cerebral artery was 350 ± 135 mg (n = 9). Vehicle (DMSO) had no significant effects on the contraction by oxyhemoglobin (Fig. VII-3a). Glibenclamide (10 μM) fully relaxed the contraction by oxyhemoglobin (Fig. VII-3b). Rings of cerebral artery which had been incubated with glibenclamide (10 μM) for 15 min were substantially less responsive to oxyhemoglobin (10 μM; Fig. VII-4). The reduction was from 350 ± 106 mg in control preparations to 125 ± 87 mg, a reduction in excess of 60%. The relaxation is dose-related as shown in Fig. VII-5.

DISCUSSION

There have been few reports about the effects of glibenclamide on responses to eicosanoids. Glibenclamide decreased plasma levels of thromboxane B_2 and β-thromboglobin in non-insulin-dependent diabetics, but there was no change in 6-keto-prostaglandin $F_{1\alpha}$, the stable metabolite of prostacyclin (Brunner et al., 1984). Glibenclamide and some other sulfonylureas reportedly enhance the release of prostaglandin I_2 in rat thoracic aorta in vitro (El Tahir et al., 1986). Glibenclamide selectively and competitively inhibited the contractile responses of canine coronary artery to U46619, an analogue of thromboxane A_2 (Cocks et al., 1990).

We have reported previously that glibenclamide relaxed the contractions induced by prostaglandin $F_{2\alpha}$ in canine cerebral and peripheral arteries and in rat aorta. These actions of glibenclamide were neither endothelium-dependent nor closely related to activation of guanylate cyclase (Zhang et al., 1991). We have shown here that glibenclamide had no significant effect on the contractions induced by KCl, caffeine and NA in canine middle cerebral artery, but significantly inhibited the contractile responses of canine middle cerebral artery to prostaglandin $F_{2\alpha}$. Glibenclamide may inhibit the responses to low concentrations of 5-HT, but the effect does not reach statistical significance and is not evident at all when higher doses of 5-HT are examined.

The mechanism by which glibenclamide produces relaxation of smooth muscle is

not clear. Since the opening of potassium channels causes relaxation in vascular smooth muscle (Standen et al., 1989), it would be expected that glibenclamide would inhibit such relaxation if its action depends on blockade of ATP-dependent potassium channels. On the other hand, in the studies reported here, glibenclamide actually causes a relaxation, rather than inhibits it. Thus we can conclude that blockade of ATP-dependent potassium channels is unlikely to be the mechanism by which this agent relaxes eicosanoid-induced contractions. Since neither the KCl-induced contraction which largely results from influx of extracellular calcium through voltage-dependent calcium channels, nor the effects of caffeine which releases calcium from intracellular stores, were affected by glibenclamide, effects on these systems can probably be ruled out. Turning to intracellular second messenger system, the response to NA is probably mediated in middle cerebral arteries by activation of α_2 receptors (Toda, 1983) which couple through the inhibitory G-protein G_i to adenylate cyclase and cause an inhibition of the enzyme (Bylund, 1988). This response is unaffected by glibenclamide, and this observation, coupled with the report of Willenbucher et al. (1991) that the cAMP-induced relaxation of the circular muscle of the distal portion of rabbit colon is unaffected by glibenclamide, suggest that the antagonist does not directly influence the adenylate cyclase system. Finally the effects of 5-HT are probably mediated by S_2 receptors which couple to phospholipase C to produce an elevation of intracellular inositol (1,4,5)-trisphosphate which, in turn, causes a release of intracellular calcium (Abdel-Latif, 1986). A similar mechanism seems to operate for the eicosanoid receptors, and our observation that the latter is blocked but the former is not significantly affected, suggests that the action of glibenclamide may not be mediated through the inositide pathway. A recent report by Yoshitake et al. (1991), however, suggests that glibenclamide may decrease intracellular calcium in rabbit aorta. They found some decrease in the response to KCl and NA produced by glibenclamide, while in cerebral arteries, we find the response to these agents is unaffected.

Glibenclamide (1-10 μM) fully relaxed the sustained precontractions induced by

prostaglandin $F_{2\alpha}$, prostaglandin D_2, prostaglandin E_2 and U46619. At a similar concentration, pre-incubation or subsequential application of glibenclamide markedly inhibited the contraction of canine middle cerebral artery to oxyhemoglobin. These results, together with some other findings that glibenclamide had no effects on KCl, NA, endothelin in canine coronary artery (Cocks et al., 1990), angiotensin II in rabbit aorta (Quast and Cook, 1989), caffeine in rabbit renal artery (Wilson and Cooper, 1989) or acetylcholine in guinea pig coronary artery (Eckman and Keef, 1991) suggest that glibenclamide is a selective inhibitor of eicosanoid-induced contractions or at least those contractions which arise from products of the cyclooxygenase pathway. These results further suggest that the contractions induced by oxyhemoglobin in isolated canine middle cerebral artery may be caused, at least in part, by release of eicosanoids. The obvious suggestion is that glibenclamide is a rather non-selective antagonist of eicosanoid receptors, but although our results are consistent with this view, it certainly cannot be regarded as established beyond doubt.

Oxyhemoglobin is now believed to be the principal cause in the development of cerebral vasospasm after subarachnoid hemorrhage (Weir, 1987). The mechanism by which oxyhemoglobin produces vasospasm is still unclear, although there is evidence that oxyhemoglobin may exert its effects by causing release of eicosanoids (Okamoto et al., 1984). It is reported that arachidonic acid is found in an esterified form in cell membrane phospholipids, from which it can be liberated through multiple enzymatic pathways (Piomelli and Greenagard, 1990). It can then diffuse out of the cell, be reincorporated into phospholipids, or undergo metabolism. For example, the cyclooxygenase product thromboxane A_2, is released from blood platelets and acts both on the platelets themselves and on vascular smooth muscle (in both cases via a membrane receptor) (Shimizu and Wolfe, 1990). There are suggestions that oxyhemoglobin stimulates intracellular production of prostaglandin $F_{2\alpha}$ in smooth muscle, and that the prostaglandin $F_{2\alpha}$ is carried outside to react with its own receptor to cause contraction of the same muscle cell (Doi et

al., 1989). A novel thromboxane A_2/prostaglandin endoperoxide receptor antagonist, ONO-3708 inhibits canine basilar arterial contractions induced by 9,11-epithio-11,12-methano-thromboxane A_2, 15-Hydroperoxyeicosatetraenoic acid (15-HPETE), U46619 or prostaglandin $F_{2\alpha}$ without affecting the contractions induced by angiotensin II, serotonin or norepinephrine in rabbit aorta (Kondo et al., 1989). In the same study, the authors found that ONO-3708 prevented cerebral vasospasm in an experimental subarachnoid hemorrhage model in dogs.

There is other evidence of a role for eicosanoids in production or maintenance of vasospasm (Weir, 1987). OKY 046, which inhibits TXA_2 synthetase, diminished vasospastic effects of whole blood in rabbit basilar arteries and in an in vivo dog model of cerebral vasospasm (Komatsu et al., 1986). Both Toda (1990) and Lang and Maron (1988) suggested that a variety of cyclooxygenase inhibitors could interfere with the effects of oxyhemoglobin or whole blood, although these agents are ineffective in clinical vasospasm in man. Cerebrospinal fluid from patients with cerebrovascular spasm, does apparently contain elevated levels of prostaglandins, particularly prostaglandin D_2 (Gaetani et al., 1986) and prostaglandin $F_{2\alpha}$ (Chehrazi et al., 1989).

Thus the ability of glibenclamide to block both the prostaglandins and oxyhemoglobin, not only suggests a mechanistic link between these spasmogens, but also implies that glibenclamide may be of some interest in the management of clinical vasospasm. Studies in a whole animal model of vasospasm will be necessary, however, to confirm this effect.

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Table VII-1. pD_2 values and maximum contractions (mg) induced by KCl, NA, 5-HT and prostaglandin $F_{2\alpha}$ in the presence and absence of glibenclamide (n=6).

	Conc	KCl		NA		5-HT		$PGF_{2\alpha}$	
Drug	[μM]	Max	pD_2	Max	pD_2	Max	pD_2	Max	pD_2
Glibenclamide	0	1015	19	221	6.4	850	7.4	2600	5.1
	1	1056	19	198	6.6	866	7.3	2200	5.0
	10	1081	22	191	6.6	754	7.3	2400	4.5

Data were fit according to the method of Zhang et al., (1991). Max was the maximum contraction obtained (mg). pD_2 is -log concentration of agonist which produced half-maximal contraction.

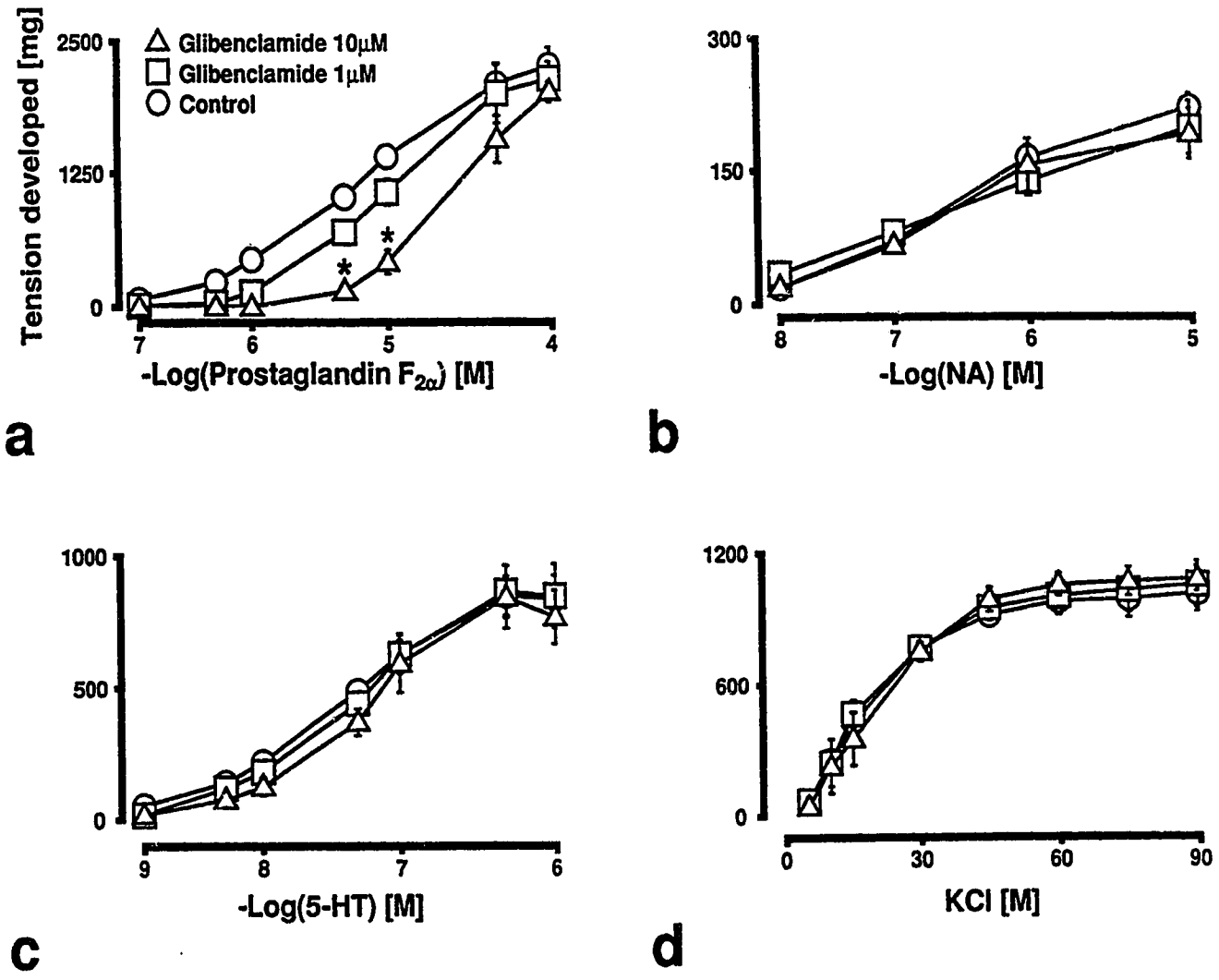

Figure VII-1. Effects of glibenclamide (○, 0 μM; □, 1 μM; Δ, 10 μM) on responses of canine middle cerebral arteries to prostaglandin $F_{2\alpha}$ (a), NA (b), 5-HT (c) and KCl (d). Bars represent standard errors of 6 rings. Asterisks represent a response significantly different from control.

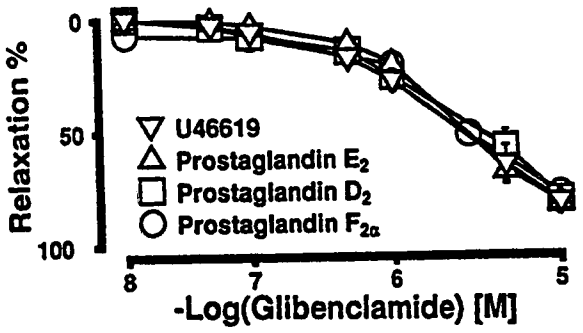

Figure VII-2. Effects of glibenclamide on the sustained contractions to prostaglandin $F_{2\alpha}$ (○, 3 μM), D_2 (□, 3 μM), E_2 (△, 3 μM) and U46619 (▽, 1 nM). Bars represent standard errors of 4 rings. Relaxation was expressed as a percentage of the response to papaverine (100 μM).

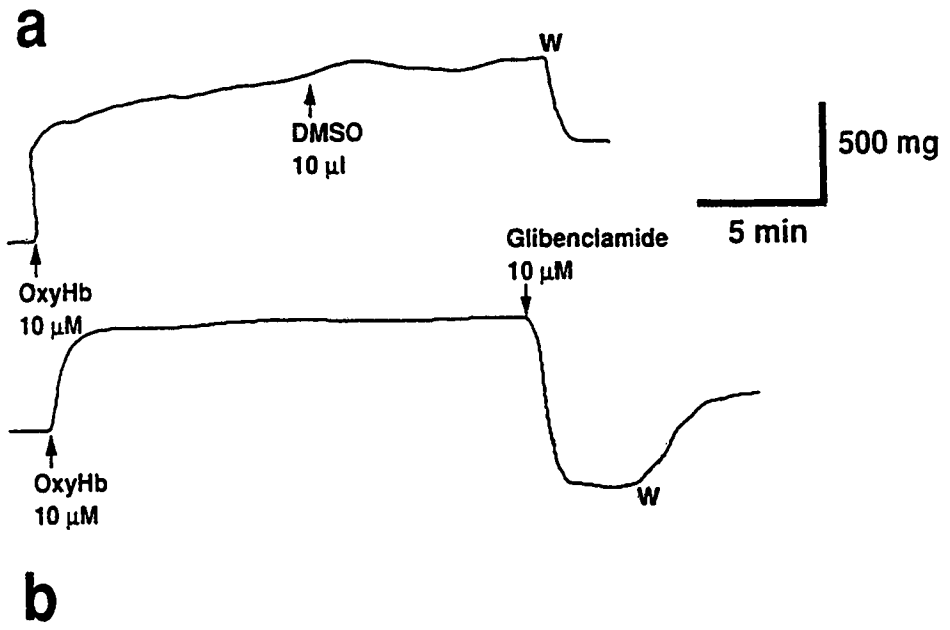

Figure VII-3. a) Contraction of canine middle cerebral artery to oxyhemoglobin (10 µM); vehicle (DMSO) had no relaxant effects on the contraction by oxyhemoglobin (10 µM); b) glibenclamide (10 µM) fully relaxed the contraction by oxyhemoglobin (10 µM). Part of the contractions were recovered after wash. The similar results were observed in another 9 rings.

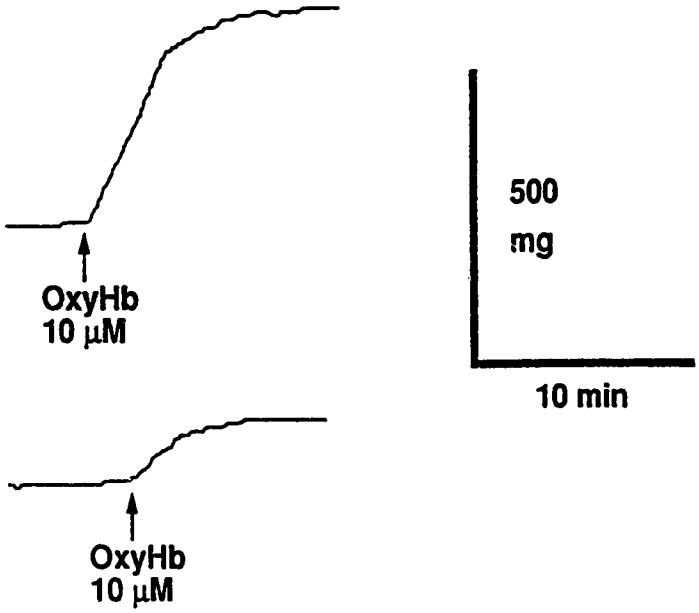

Figure VII-4. Contraction by oxyhemoglobin (10 μM) in the absence of glibenclamide (top); After pre-incubation with glibenclamide (10 μM) for 15 min, oxyhemoglobin (10 μM) induced less contraction in the same ring (bottom). The similar results were observed in another 3 rings.

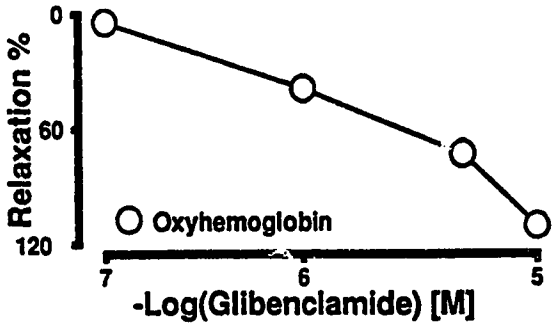

Figure VII-5. Dose-response curve for the effects of glibenclamide on the contraction induced by oxyhemoglobin (10 μM) in canine middle cerebral artery. Bars represent standard errors of 9 rings. Relaxation was expressed as a percentage of the stable response to oxyhemoglobin.

CHAPTER EIGHT

ANTAGONISM OF EICOSANOID-INDUCED CONTRACTION OF RAT AORTA BY SULPHONYLUREAS

SUMMARY

The inhibitory effect of sulphonylureas on the contractions of rat aorta induced by prostaglandins, norepinephrine, 5-hydroxytryptamine (5-HT) or potassium chloride (KCl) was examined. (1) Glibenclamide significantly inhibted the contractions produced by prostaglandins $F_{2\alpha}$, E_2 and D_2, but was without effect on responses to norepinephrine, 5-HT or KCl. (2) Glimepiride produced significant attenuation of the responses to all agents tested (5-HT, norepinephrine, KCl), although inhibition of the responses to prostaglandin $F_{2\alpha}$ was most pronounced. Glipizide and tolbutamide had activities similar to that of glibenclamide, while chlorpropamide was devoid of any antagonist action in rat aorta. (3) In rat aorta pre-contracted with norepinephrine, glibenclamide inhibited the relaxant responses to lemakalim and pinacidil. The action of nicorandil was not reversed by glibenclamide but this response was affected by methylene blue, an agent which did not affect the responses to pinacidil or lemakalim. Thus, in addition to effects on ATP-dependent potassium channels, glibenclamide inhibits responses to prostaglandins $F_{2\alpha}$, E_2 and D_2 but not to other agents. This effect is shared by glimepiride, tolbutamide and glipizide.

INTRODUCTION

The secretion of insulin from pancreatic islet β-cells is modulated by ATP-dependent potassium channels. Glucose, as well as the sulphonylureas, induces a depolarization by closing ATP-dependent potassium channels, which leads to the activation of L-type calcium channels, to calcium entry, and to insulin secretion (Weille et

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A version of this chapter has been submitted for publication. H. Zhang, B. Weir, N. Stockbridge and D. Cook. 1991. *Eur J Pharmacol*

al., 1989). The ATP-dependent potassium channels have now been identified as the functional receptors for sulphonylureas (Schmid-Antomarchi et al., 1987). In addition, the sulphonylureas can affect ATP-dependent potassium channels in other tissues, to change not only their normal activity, but also their response to drugs. The effects of potassium channel openers such as cromakalim or pinacidil are attenuated by the sulphonylureas, either in cardiac myocytes (Sanguinetti et al., 1988; Escande et al., 1989a) or in vascular smooth muscle (Standen et al., 1989).

In addition to the action of the sulphonylureas on ATP-dependent potassium channels, glibenclamide itself, which is a clinically important compound of this class, has other actions in vascular preparations. Glibenclamide was shown to be a competitive antagonist at thromboxane A_2 receptors in canine coronary arteries (Cocks et al., 1990) and to antagonize contractions to prostaglandin $F_{2\alpha}$ in rat aorta and canine cerebral and peripheral arteries (Zhang et al., 1991a). The effects of glibenclamide are not dependent on cyclic guanosine monophosphate (cGMP) nor on the presence of intact endothelium (Zhang et al., 1991a). The mechanism by which these effects are produced is not clear. Because prostaglandin $F_{2\alpha}$ uses similar signal transduction mechanism to agents such as norepinephrine, which is not antagonized by glibenclamide (Cocks et al., 1990), it is more likely that glibenclamide acts early in the stimulus-response sequence rather than on subsequent intracellular processes. Chappell et al. (1991) suggested that glibenclamide might act as a calcium antagonist in rat pulmonary artery, since it antagonized contractions induced by KCl, although there seems no other evidence for effects on calcium conductance (Schmid-Antomarchi et al., 1987), and the apparent selectivity of this action for eicosanoids argues against such a non-specific mechanism. Also it is not clear whether the ability to block ATP-dependent potassium channels is necessary or sufficient for effects on eicosanoid responses. We have thus examined the responses of isolated rings of rat aorta to provide information about the selectivity of glibenclamide for the responses to different prostaglandins and the effects of different sulphonylureas on the responses to prostaglandin

$F_{2\alpha}$. We have examined the inhibitory action of glibenclamide on the relaxations produced by the potassium channel openers, nicorandil, pinacidil and lemakalim (BRL 38227), the vasorelaxant (-)-trans enantiomer of cromakalim (Edwards and Weston, 1990), to determine whether action at ATP-dependent potassium channels can be dissociated from inhibitory effects on eicosanoid-produced contractions.

MATERIALS AND METHODS

Preparation of Aortic Rings

Sprague-Dawley rats (300-400 g) were killed by exposure to halothane. The aorta was removed and immersed immediately in Krebs' physiological saline solution equilibrated with 95% O_2 and 5% CO_2 at room temperature. The composition of the medium was (mM): NaCl 113.7, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and dextrose 10.1. Arteries were mounted vertically between small hooks in water-jacketed tissue baths of 10 ml working volume maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). No attempt was made to remove the endothelium.

Arterial Tension Recordings

At the beginning of each experiment, the rings were stretched to an initial tension of 1 g and allowed to equilibrate for approximately 2 h. During the equilibration period, the bathing medium was changed at 15-20 min intervals to prevent the accumulation of metabolites. Contractions and relaxations were recorded isometrically using strain gauges (model FTO 3, Grass Instrument Co.) connected to a polygraph (model 7D, Grass Instrument Co.).Concentration-response curves for the contractions produced by KCl, norepinephrine, 5-HT and prostaglandins $F_{2\alpha}$, D_2 and E_2 were obtained. The tissues were washed and allowed to recover for 1.5 hour, and a second concentration-response curve was then obtained in the presence of 1 or 10 μM glibenclamide or of 10 or 100 μM tolbutamide, chlorpropamide, glimepiride or glipizide. All the antagonists were added to the bath 15 min before the addition of agonists.

In another series of experiments, the preparations were contracted with

norepinephrine (0.1 μM, approximately the EC_{50}) in the absence or in the presence of glibenclamide (20 μM, 15 min pre-incubation). When a stable contraction had been obtained, cumulative concentrations of nicorandil, pinacidil and lemakalim were administered. The relaxant response to each concentration was allowed to reach a plateau before addition of the next dose. At the end of each experiment, papaverine (100 μM) was applied to produce a maximum relaxation. In some arteries, contractions were induced by prostaglandin $F_{2\alpha}$ (3 μM, approximately the EC_{50}) in the absence and presence of methylene blue (3 μM) and nicorandil, pinacidil and lemakalim were applied as mentioned above.

Drugs and Solutions

Glipizide and chlorpropamide were obtained from Pfizer Canada, and glimepiride from Hoechst AG (Federal Republic of Germany). Potassium channel openers were gifts from Leo (pinacidil), Upjohn (nicorandil) and Beecham (lemakalim). All other drugs were obtained from Sigma.

A stock solution of 10 mM glibenclamide was made in dimethyl sulfoxide. Other sulphonylureas and potassium channel openers were dissolved in 95% ethanol. Stock solutions of prostaglandins were prepared in 50% ethanol. Control experiments established that observed effects were not caused by the vehicles.

Statistical Analysis

The results were expressed as means ± standard error of the mean and compared using analysis of variance or one-tailed *t* test (BMDP Statistical Software). A probability of < 0.05 was considered significant. Maximum contractions and pD_2 values were calculated by the methods of Zhang et al. (1991a). The pD_2 values were calculated on the assumption that the concentration-response curves obtained in the presence of the antagonist are parallel to the control curves and that the control maximum response would ultimately be achieved at sufficiently high agonist concentration. This issue is discussed in more detail later.

RESULTS

Preliminary experiments were conducted to determine whether the sulphonylureas had a direct effect on resting tone. At the concentrations employed in the work reported here, none of these agents had any effects on the resting tension of the rat aorta. Equally the vehicles used to dissolve the drugs were devoid of activity at the concentrations to which the tissues were exposed during the experiments.

The inhibitory effect of glibenclamide on the contractions induced by prostaglandins and other agents.

The blocking effects of glibenclamide on the responses of rat aorta to prostaglandins $F_{2\alpha}$, D_2 and E_2 are shown in Table VIII-1 and in Fig. VIII-1. In each case there was a concentration-dependent inhibition of the effects of the prostaglandins which was significant even at a concentration of 1 μM. Effects on 5-HT, KCl and norepinephrine are shown in Fig. VIII-2; there is no significant antagonism, even at the higher concentration of glibenclamide (10 μM).

The inhibitory effect of other sulphonylureas on the contractions induced by prostaglandin $F_{2\alpha}$ and other agents.

The results obtained with the other sulphonylureas are shown in Fig. VIII-3-4 and in Table VIII-2. Glimepiride is an antagonist of responses to prostaglandins $F_{2\alpha}$ (Fig. VIII-3a) but also significantly antagonizes responses to the other agents, at least at the higher concentration (Fig. VIII-4). The maximum contractions induced by KCl, 5-HT and norepinephrine were reduced by glimepiride (100 μM), while the pD_2 values were not changed (Table VIII-2). Glipizide (100 μM; Fig. VIII-3b) and tolbutamide (100 μM; Fig. VIII-3c) behave qualitatively like glibenclamide in that the responses to prostaglandin $F_{2\alpha}$ are significantly ($p<0.05$) antagonized, but not the responses to other agents. The maximum contractions and pD_2 values remained unchanged (Table VIII-2). Chlorpropamide (Fig. VIII-3d) seems entirely devoid of effects in this system even at concentrations of 100 μM.

The inhibitory effect of glibenclamide on the relaxations produced by

potassium channel agonists.

The effects of glibenclamide on potassium channels were tested by using the potassium channel opening drugs, nicorandil, pinacidil and lemakalim. These agents relaxed the sustained contractions induced by norepinephrine (0.1 μM) in a concentration-dependent manner. Pre-incubation with glibenclamide (20 μM) did not decrease contractions induced by norepinephrine, but significantly attenuated the relaxant effects of lemakalim and pinacidil, but not nicorandil (Fig. VIII-5). Methylene blue was used to examine whether the action of these drugs is mediated by an increase in cGMP concentration. Nicorandil, pinacidil and lemakalim relaxed the sustained contractions induced by prostaglandins $F_{2\alpha}$ (3 μM). Methylene blue (3 μM) pretreatment slightly enhanced the response of rat aorta to prostaglandin $F_{2\alpha}$ and significantly decreased the effects of nicorandil but not pinacidil and lemakalim (Fig. VIII-6).

DISCUSSION

The observation that glibenclamide antagonizes responses to prostaglandin $F_{2\alpha}$ is in agreement with previous findings in canine cerebral arteries and in rat aorta (Zhang et al., 1991a). Cocks et al. (1990) reported similar findings in canine coronary arteries, although they used the thromboxane A_2 analogue, U46619, rather than prostaglandin $F_{2\alpha}$, and found that this response was sensitive to glibenclamide while the responses to a variety of other agonists were unaffected. In addition to antagonizing the action of eicosanoid, glibenclamide can also antagonize the responses to norepinephrine and KCl in rabbit aorta (Yoshitake et al., 1991). Chappell et al. (1991) reported similar findings in rat pulmonary artery, and suggested that glibenclamide might have the ability to block calcium channels. Another possble explanatin for the relaxation of glibenclamide in vascular smooth muscle is that glibenclamide increases the release of prostacyclin (El Tahir et al., 1986). Even though there is a suggestion that glibenclamide, at high concentrations (> 10 μM), opens potassium channels in vascular smooth muscle (Hu er al., personal communication), other studies using the similar concentration did not reveal the same observation. It is possible

that these differences arise from factors of tissue, species, experimental methods or concentration of antagonists, but at least in the tissue we have examined, there is little doubt that glibenclamide antagonizes contractions elicited with prostaglandin $F_{2\alpha}$ under circumstances where responses to KCl, norepinephrine or 5-HT are not significantly affected.

The data from Cocks et al. (1991) and our own earlier results suggest that this action may be associated with receptors for eicosanoids, and this view is supported by our observation that responses to prostaglandin D_2 and prostaglandin E_2 are also sensitive to glibenclamide, at least in rat aortic ring preparations. The nature of the antagonism of eicosanoid responses produced by the sulphonylureas in not clear. The largest responses obtained in the presence of the sulphonylureas, are frequently significantly smaller than the maximum control response, particularly at the higher concentration of the antagonists. It seems probable, however, that the maximum responses in the presence of the antagonist have not been achieved at the highest concentration of agonist actually tested. This is an assumption implicit in the method used to estimate the pD_2, in that the maximum response in the presence of the antagonist is taken to be the same as that of control preparation. This approach is far from satisfactory, but to use the eicosanoids at concentrations in excess of 100 μM is equally unrealistic. This assumption was supported by our preliminary results obtained from canine middle cerebral arteries, which had showed that the maximum responses in the presence of glibenclamide reaches the same level as control, while the concentration-response curves were shifted to right, suggesting that glibenclamide competitively inhibits the contractile response to prostaglandin $F_{2\alpha}$ (Zhang et al., unpublished observation). The data shown in table 2 provides both the maximum response to the highest agonist concentration tested, and the pD_2 based on the assumptions outlined above. These data provide convincing evidence that the control response is blocked, but cannot be interpreted to define the nature of the antagonism. Examination of the concentration-response curves in Fig. VIII-1 suggest that the antagonism is probably

competitive, but this certainly not established beyond doubt.

The mechanism by which glibenclamide antagonizes the action of the eicosanoids is far from clear, but it is probably not at the level of the signal transduction process. Norepinephrine, through its action at the α_1 receptor, and 5-HT via the 5-HT_2 receptor, produce contraction through a G-protein mediated activation of phospholipase C, with a consequent elevation of inositol (1,4,5)-trisphosphate, which interacts with a receptor on the endoplasmic reticulum to release calcium from intracellular sites. The eicosanoids seem to share this mechanism of action (Abdel-Latif, 1986) but are sensitive to glibenclamide, while norepinephrine and 5-HT are not. While it has been suggested by a variety of authors that the sulphonylureas have some action on eicosanoid synthesis and release (Brunner et al., 1984; El Tahir et al., 1986; Goldraj et al., 1987; Florkowski et al., 1988), these effects are not likely to play a role in the results obtained here, where the eicosanoids are simply added to the organ bath rather than released from the preparation itself.

In order to determine whether this property is shared by other sulphonylureas, we examined the effects of glipizide, glimepiride, tolbutamide and chlorpropamide. Glipizide and tolbutamide had actions which were similar to glibenclamide in that contractions to prostaglandin $F_{2\alpha}$ were antagonized, while responses to KCl, 5-HT or norepinephrine were not significantly affected, while chlorpropamide was without effect at any concentration tested. Glimepiride has antagonist action against the responses to prostaglandin $F_{2\alpha}$, but at the highest dose tested also antagonized responses to KCl, norepinephrine and 5-HT although to a lesser extent than those to prostaglandin $F_{2\alpha}$. The inhibition of the contractions to KCl, norepinephrine and 5-HT by glimepiride seems in a non-competitive manner, since the maximum response was depressed while pD_2 values were not changed. The approximate order of potency for actions against contractions of rat aortic ring preparations to prostaglandin $F_{2\alpha}$ was glibenclamide > glimepiride > glipizide > tolbutamide >> chlorpropamide (Table VIII-1). The order of the therapeutic potency of the sulphonylureas is glimepiride > glibenclamide > glipizide > chlorpropamide > tolbutamide

(Schmid-Antomarchi et al., 1987; Geisen, 1988), while the order of potency at blocking ATP-dependent potassium conductance in isolated β-cells is glibenclamide > glipizide > tolbutamide ≅ chlorpropamide (Schmid-Antomarchi et al., 1987). Since the clinical efficacy is a function of both an action at potassium channels as well as absorption, metabolism and protein binding, it appears that the therapeutic potency, and the relative potency on potassium channels are close to that obtained from the present study on prostaglandin $F_{2\alpha}$.

While the issue of the ability of the sulphonylureas to antagonize the action of the eicosanoids is apparently distinct from their action at ATP-dependent potassium channels, the question of whether the latter mechanism plays any role in vascular smooth muscle is still of interest. Since activation of ATP-dependent potassium channels would be expected to lead to relaxation in vascular smooth muscle, in order to investigate the phenomenon it is necessary first to contract the preparation and norepinephrine was used for this purpose. As expected, nicorandil, pinacidil and lemakalim all caused relaxation, and in the case of pinacidil and lemakalim, this effect was completely inhibited by glibenclamide. Nicorandil was not antagonized by glibenclamide, but in tissues precontracted with prostaglandin $F_{2\alpha}$, the effect was antagonized by methylene blue which inhibits guanylate cyclase (Greutter et al., 1981). Methylene blue did not inhibit the actions of pinacidil or lemakalim. It thus seems that glibenclamide can inhibit the opening of potassium channels produced by pinacidil (also see Arena and Kass, 1989) and lemakalim (see Sanguinetti et al., 1988), but that in rat aorta, the relaxation produced by nicorandil depends mainly on its ability to generate cyclic GMP rather than effects on potassium channels. This observation is in agreement with a report that nicorandil does not activate potassium channels in rat basilar arterial smooth muscle while pinacidil and cromakalim do (Zhang et al., 1991b).

Thus, in summary, in vascular smooth muscle many sulphonylureas can interfere with responses produced by a variety of eicosanoids. This action seems to arise from effects on an early stage of the stimulus-response mechanism. In addition, glibenclamide can

inhibit the relaxation produced by pinacidil and lemakalim in the same preparation, presumably by interfering with the ability of these compounds to open ATP-dependent potassium channels. The relaxation produced by nicorandil appears to result from effects on guanylate cyclase, and is not sensitive to glibenclamide.

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Table VIII-1. Effects of glibenclamide on pD_2 values and maximum contractions (g) of prostaglandins $F_{2\alpha}$, D_2 and E_2 in rat aorta

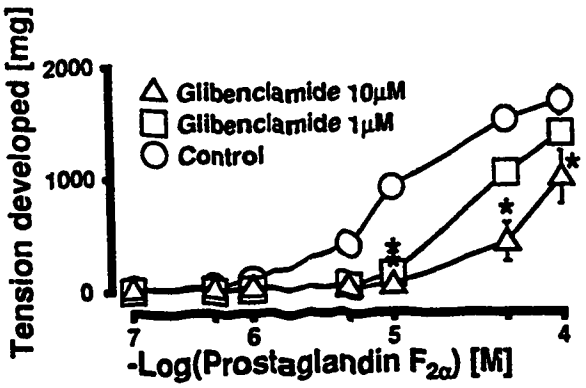
Drug	Conc [μM]	$PGF_{2\alpha}$ R_{max}	$PGF_{2\alpha}$ pD_2	PGD_2 R_{max}	PGD_2 pD_2	PGE_2 R_{max}	PGE_2 pD_2
glibenclamide	0	1.8	5.0	1.8	4.4	2.0	4.7
	1	1.6	4.4	1.7	4.2	1.5	3.9
	10	1.0	3.6	0.7	3.8	0.6	3.8

The observed data were fitted by the method of Zhang et al. (1991a) to provide estimates of the pD_2 value, obtained using the assumption that the maximum response was the same in the presence of the antagonist as in its absence. $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; PGD_2 = prostaglandin D_2; PGE_2 = prostaglandin E_2; Conc = concentration; R_{max} = maximum response. The R_{max} and pD_2 values for $PGF_{2\alpha}$, PGD_2 and PGE_2 were calculated from 5-8 experiments.

Table VIII-2. Effects of sulphonylureas on pD_2 values and maximum contractions (g) of KCl, norepinephrine, 5-HT and prostaglandin $F_{2\alpha}$ in rat aorta

	Conc	KCl		NE		5-HT		$PGF_{2\alpha}$	
Drug	[μM]	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2	R_{max}	pD_2
chlorpropamide	0	1.2	1.4	1.5	7.7	1.2	5.2	1.9	4.9
	10	1.3	1.3	1.6	7.7	1.3	5.2	2.0	4.7
	100	1.3	1.3	1.6	7.6	1.1	5.3	1.9	4.6
tolbutamide	0	1.1	1.6	1.7	7.9	1.4	5.5	1.5	5.2
	10	1.2	1.5	1.7	7.9	1.2	5.3	1.6	5.1
	100	1.0	1.5	1.6	7.6	1.2	5.2	1.3	4.6
glipizide	0	1.2	1.6	1.3	7.9	1.3	5.5	1.9	5.2
	10	1.3	1.5	1.3	8.0	1.3	5.2	2.0	5.0
	100	1.1	1.6	1.2	7.6	1.0	5.3	1.4	4.1
glimepiride	0	1.2	1.6	1.4	7.6	1.3	5.6	1.7	5.0
	10	1.2	1.6	1.3	7.5	1.3	5.4	1.6	4.7
	100	0.8	1.5	1.0	7.3	0.8	5.3	0.4	3.0
glibenclamide	0	1.1	1.6	1.6	7.5	1.5	5.6	1.8	5.0
	1	1.1	1.5	1.5	7.6	1.4	5.5	1.6	4.4
	10	1.1	1.6	1.4	7.7	1.4	5.4	1.0	3.6

The observed data were fitted by the method of Zhang et al. (1991a). The pD_2 values of prostaglandin $F_{2\alpha}$ were obtained using the assumption that the maximum response was the same in the presence of the antagonist as in its absence. $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; NE = norepinephrine; Conc = concentration; R_{max} = maximum response. The R_{max} and pD_2 values for KCl, NE and 5-HT were calculated from 4-9 experiments.



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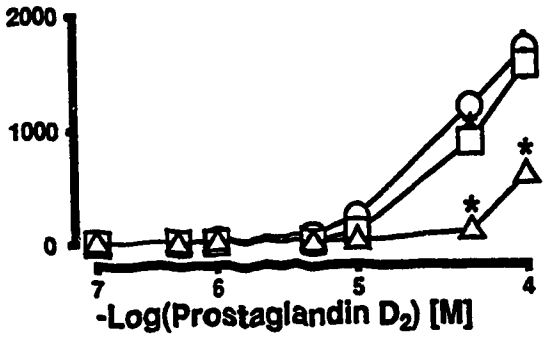

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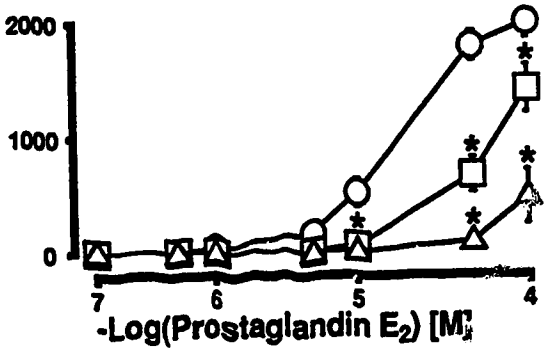

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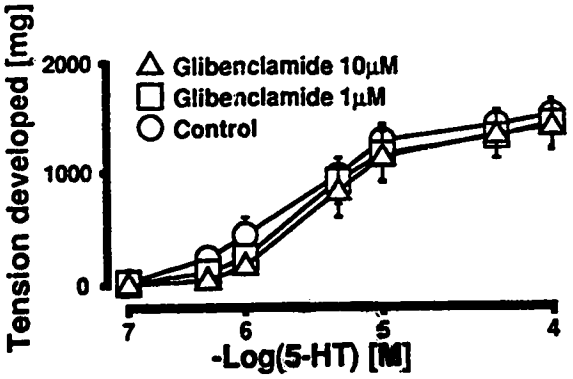
Figure VIII-1. Effects of glibenclamide on the contractile responses of rat aorta to prostaglandin $F_{2\alpha}$ (n=5-8), D_2 (n=6) and E_2 (n=4). Bars represent standard errors of the means and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of glibenclamide. Glibenclamide (1 μM (□) or 10 μM (△)) was pre-incubated for 15 min before agonists were administered.



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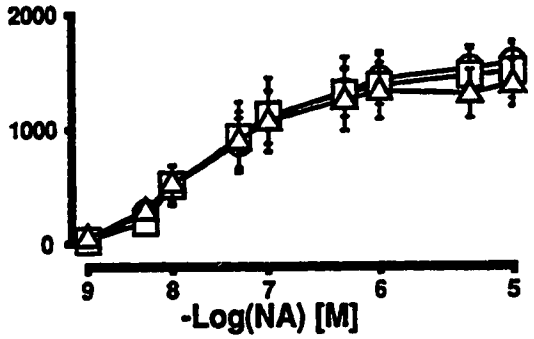

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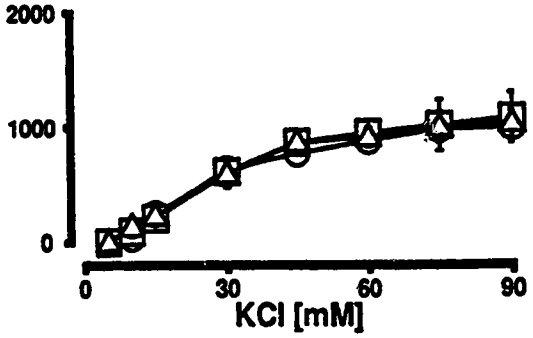

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Figure VIII-2. Effects of glibenclamide on the contractile responses of rat aorta to 5-HT (n=6), norepinephrine (n=6) and KCl (n=7). Bars represent standard errors of the means and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of glibenclamide. Glibenclamide (1 μM (□) or 10 μM (△)) was pre-incubated for 15 min before agonists were administered.

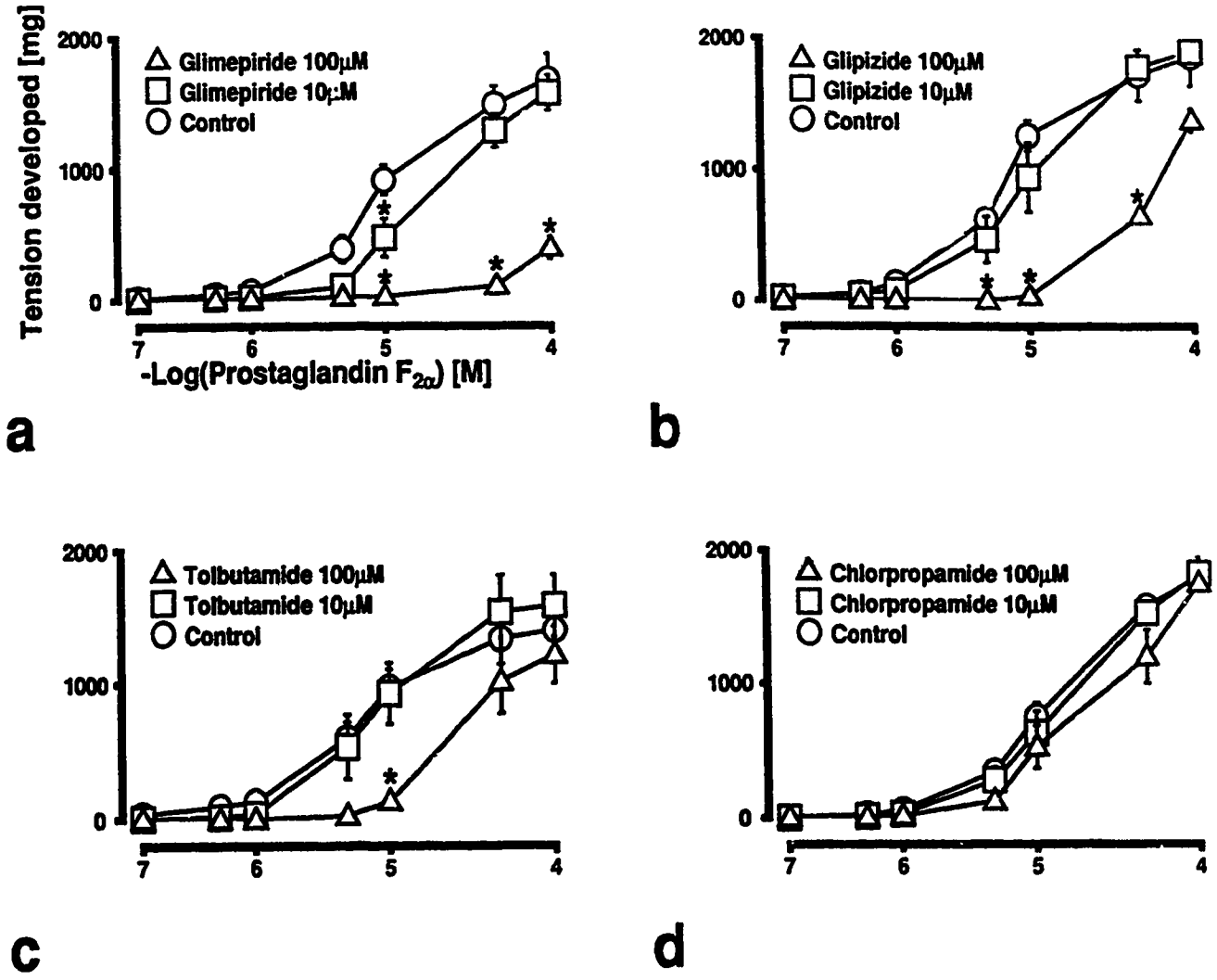
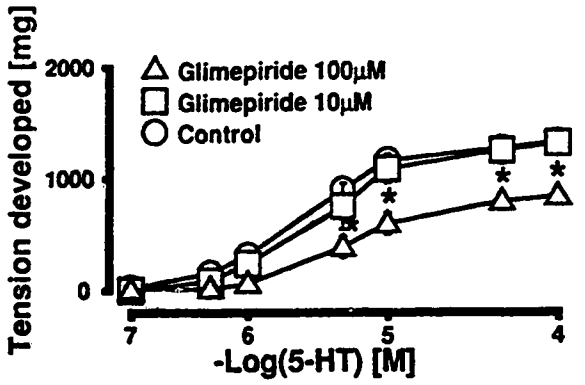


Figure VIII-3. Effects of sulphonylureas glimepiride (a), glipizide (b), tolbutamide (c) and chlorpropamide (d) on the contractile responses of rat aorta to prostaglandin $F_{2\alpha}$. Bars represent standard errors of the means of six experiments and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of sulphonylureas. Sulphonylureas (10 μM (□) or 100 μM (△)) was pre-incubated for 15 min before prostaglandin $F_{2\alpha}$ were administered.



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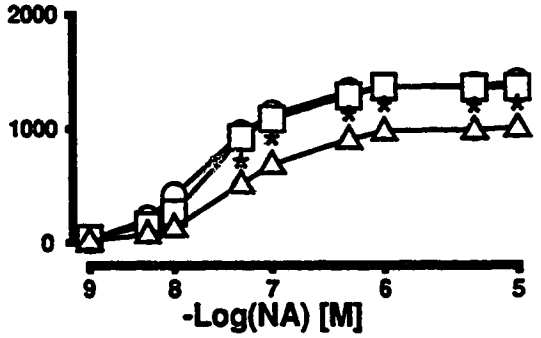

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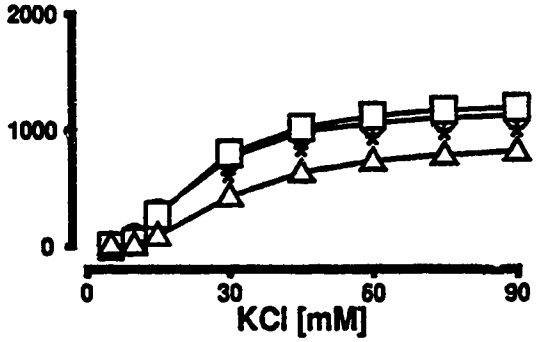

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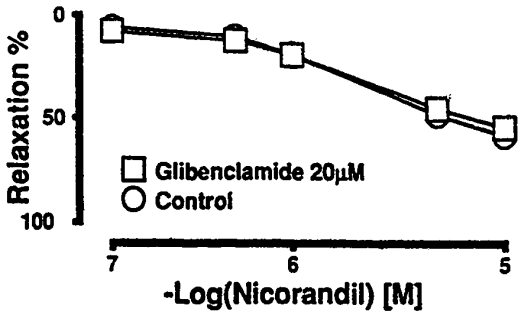
Figure VIII-4. Effects of glimepiride on the contractile responses of rat aorta to 5-HT (n = 4-8), norepinephrine (n = 5-9) and KCl (n = 5-9). Bars represent standard errors of the means and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of glimepiride. Glimepiride (10 μM (□) or 100 μM (△)) was pre-incubated for 15 min before agonists were administered.



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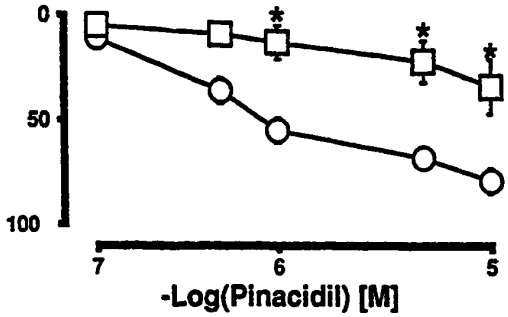

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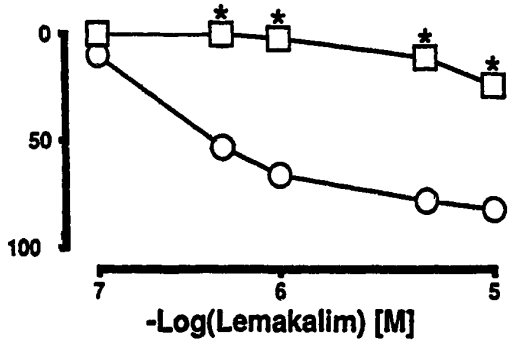
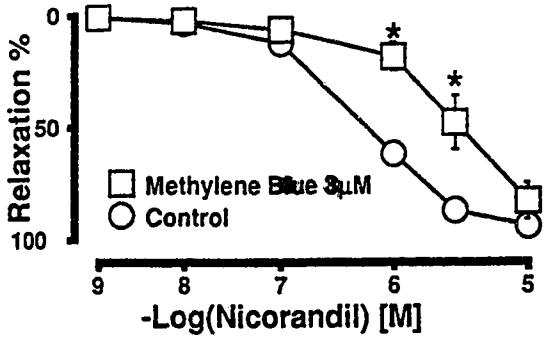


Figure VIII-5. Effects of glibenclamide on the relaxation produced by nicorandil, pinacidil and lemakalim in rat aorta. The relaxant effects of nicorandil (a), pinacidil (b) and lemakalim (c) were tested on the sustained contractions induced by norepinephrine (0.1 μM) in the absence (○) and presence (□) of 20 μM glibenclamide. Glibenclamide was pre-incubated for 15 min before potassium channel openers were administered. Bars represent standard errors of the mean for four rings and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of glibenclamide.



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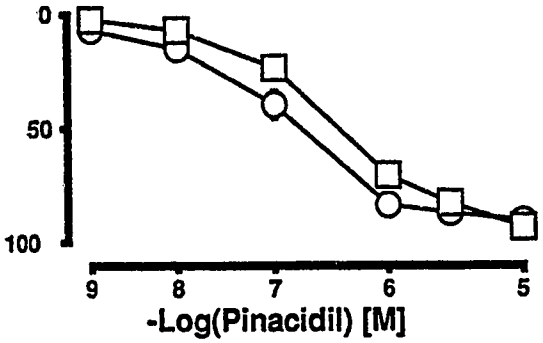

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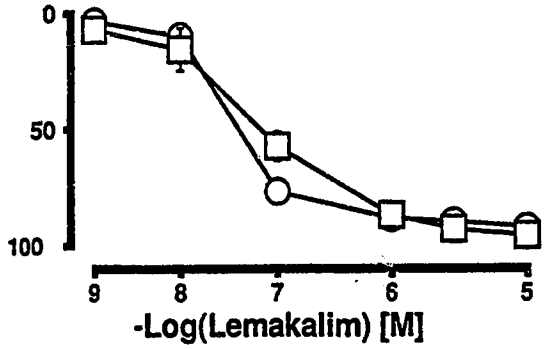

Figure VIII-6. Effects of methylene blue on the relaxation produced by nicorandil, pinacidil and lemakalim in rat aorta. The relaxant effects of nicorandil (a), pinacidil (b) and lemakalim (c) were tested on the sustained contractions induced by prostaglandin $F_{2\alpha}$ (3 µM) in the absence (○) and presence (□) of methylene blue (3 µM). Methylene blue was pre-incubated for 15 min before potassium channel openers were administered. Bars represent standard errors of the mean for four rings and asterisks mark responses significantly (* $p < .05$) different from control (○) obtained in the absence of methylene blue.

CHAPTRE NINE

RELAXANT EFFECTS OF ILOPROST IN CANINE CEREBRAL ARTERY

SUMMARY

Iloprost caused relaxation of rings of canine cerebral arteries precontracted with prostaglandin $F_{2\alpha}$ or the thromboxane A_2 analogue U 46619, but was without effect on arteries precontracted with potassium chloride. Pretreatment with iloprost did not significantly affect the concentration-response curve to any agent. Contractile responses to oxyhemoglobin were completely relaxed by iloprost. In arteries from animals with moderate cerebrovascular spasm, the response to prostaglandin $F_{2\alpha}$ was also reduced by iloprost. The observation that iloprost relaxes the response to oxyhemoglobin and prostaglandin $F_{2\alpha}$ in spastic arteries may be of interest in the management of cerebral vasospasm.

INTRODUCTION

Numerous studies have demonstrated that iloprost, an analog of prostaglandin I_2 (Schillinger et al., 1986), can produce either contraction (Vermue et al., 1987), no effect (Vermue et al., 1987) or relaxation (Siegel et al., 1989) of a wide range of smooth muscle preparations from various species. Indeed, even within one species, it can produce different results in different preparations (Demirel and Turker, 1989). In cerebral arteries, iloprost (or prostacyclin) usually causes relaxation (Parsons and Whalley, 1989; Shimokawa et al., 1988; Whalley et al., 1989), but in canine and human basilar arteries, the response is more complex and a biphasic response is often seen (Chapleau and White, 1979; Katusic et al., 1988; Parsons and Whalley, 1989). The mechanism of the effect of iloprost in smooth muscle is complicated. Iloprost activates adenylate cyclase and increases

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intracellular cAMP (Mene and Dunn, 1988), but it also modulates calcium entry into the smooth muscle cell by voltage-operated calcium channels (Demirel and Turker, 1989). Siegel et al. (1989) suggested that iloprost hyperpolarizes the cell membrane and reduces the tension by opening potassium channels.

Since oxyhemoglobin and possibly prostaglandins may play a role in the development of vasospasm after subarachnoid hemorrhage (Findlay et al., 1991), and iloprost reverses the experimental cerebral vasospasm in rabbit basilar artery (Egemen et al., 1988), we have examined the effect of iloprost on normal arteries and on spastic arteries from a canine model of vasospasm. Contraction was induced by oxyhemoglobin, prostaglandin $F_{2\alpha}$, U 46619, 5-hydroxytryptamine (5-HT) or potassium chloride (KCl). The relaxant effect of iloprost on the sustained contraction was compared to that achieved with glyceryl trinitrate, an activator of guanylate cyclase, while effects on the contraction induced by KCl were compared with nimodipine, which inhibits voltage-dependent calcium channels.

Materials and Methods

Mongrel dogs of either sex, 15-25 kg in weight, were killed by intravenous overdose of sodium pentobarbital (60 mg/kg). Rings of basilar and middle cerebral arteries were prepared as described previously (Zhang et al., 1991). No attempt was made to remove the endothelium, and since care was taken in the suspension of the arterial rings, damage to the endothelium is likely to be minimal. Arteries were mounted vertically between small hooks in a water-jacketed tissue bath of 10 ml working volume maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 (pH 7.4). The composition of the medium was (mM): NaCl 113.7, $NaHCO_3$ 25.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2 and dextrose 10.1.

At the beginning of each experiment, the rings were stretched to an initial tension of 0.5 g, and allowed to equilibrate for approximately 2 h. During the equilibration period, the bathing medium was changed at 15-20 min intervals. Responses were recorded

isometrically using strain gauges transducers (Model FTO 3, Grass Instrument Co.) connected to a polygraph (Model 7D, Grass Instrument Co.). Contractions were elicited with prostaglandin $F_{2\alpha}$ (3 μM), U 46619 (1 nM), KCl (20-40 mM) or oxyhemoglobin (10 μM). When a stable response had been obtained, cumulative doses of iloprost, glyceryl trinitrate or nimodipine were administered, allowing each response to stabilize before the next addition was made. At the end of each series of experiments, papaverine (100 μM) was applied to produce the maximum relaxation. In other experiments, contractions produced by prostaglandin $F_{2\alpha}$, 5-HT and KCl were obtained first in the absence of iloprost, then the tissues were washed every 15-20 min for 1 hr, and incubated for 15 min with iloprost at a concentration of either 0.1 μM or 1 μM. Further contractions to these agonists were then recorded.

Further studies were carried out using spastic basilar arteries obtained from a "one hemorrhage" dog model of subarachnoid hemorrhage (Allen and Bahr, 1979). Briefly, autologous blood (0.5 ml/kg) was injected percutaneously into the cisterna magna after the control angiography. The dog was killed on day 7 after angiography. The diameter of these basilar arteries showed a 12% reduction, when compared with those from saline-treated controls.

Iloprost was obtained as a solution in normal saline containing a minimal amount of tris-base and ethanol, from Schering AG (Berlin, West Germany). Iloprost is freely soluble in water and was diluted with normal saline before use. U 46619 was a gift of The Upjohn Company. All other agents were purchased from Sigma. Oxyhemoglobin was produced by reduction of human hemoglobin by sodium dithionite (J.T. Baker Chemical Co.,) (Martin et al. 1985). In statistical analysis, Each response was expressed as the mean ± s.e.mean and results were compared using one-tailed t-test. A probability value of less than 0.05 was considered significant.

RESULTS

Iloprost reduced the resting tension of canine basilar and middle cerebral arteries;

at concentration of 1 μM, the resting tension was reduced by 160 ± 95 mg (n = 9) from its control value of 500 mg.

Iloprost caused relaxation when administered during the sustained contraction to prostaglandin $F_{2\alpha}$ (3 μM) or U 46619 (1 nM). These effects were dose-dependent and similar to those which were achieved with the same concentration of glyceryl trinitrate. Iloprost did not relax the responses to KCl even at 1 μM; indeed it produced a small contraction at higher concentration. Glyceryl trinitrate was somewhat effective against KCl, while nimodipine had its expected effect of significant vasodilation at lower concentrations. These results are shown for middle cerebral artery in Fig. IX-1. Results from rings of basilar artery (not shown) are essentially identical.

Cumulative application of prostaglandin $F_{2\alpha}$, 5-HT and KCl to canine middle cerebral artery produced a vasoconstriction which was dose-dependent. Pretreatment with iloprost (0.1-1 μM) was without significant effect on the contractile responses to prostaglandin $F_{2\alpha}$, 5-HT or KCl (Fig. IX-2a,b,c).

Iloprost (1 μM) fully relaxed the contractions induced by oxyhemoglobin (10 μM) in canine cerebral arteries (n = 11; Fig. IX-3). Some responses were partly restored by washing. Iloprost relaxed the sustained contraction induced by prostaglandin $F_{2\alpha}$ (3 μM) in both spastic and control arteries (Fig. IX-4).

Discussion

The proposed mechanism by which iloprost (prostacyclin) relaxes vascular smooth muscle is that iloprost binds to the prostacyclin receptor which couples to the stimulatory guanine regulatory protein (G_s) and activates the catalytic subunit of adenylate cyclase, resulting in an increased production of cAMP (Murray, 1990). Other mechanisms, however, have recently been reported (Demirel and Turker, 1989; Siegel et al., 1989), while the relaxation produced by prostacyclin in canine basilar artery has been suggested to arise from activation of Na^+-K^+ ATPase, because it is prevented both by ouabain and by use of potassium-free solution (Katusic et al., 1988). There have been few reports about the effect

of prostacyclin in canine basilar artery and the results are controversial. Prostacyclin relaxes the sustained contraction induced by prostaglandin $F_{2\alpha}$ and 5-HT in canine basilar artery with an intact endothelium (Chapleau and White, 1979), but either had no effect or produced endothelium-dependent contraction in tissues precontracted with uridine-5'-triphosphate (Katusic et al., 1988). In the present study, we showed that iloprost causes dose-dependent relaxation of the contraction of canine cerebral artery induced by prostaglandin $F_{2\alpha}$ and U 46619. There are no significant differences between the effect of iloprost in canine basilar and middle cerebral arteries. The inability of pre-incubation with Iloprost to inhibit the contractile responses of this preparation to prostaglandin $F_{2\alpha}$ and 5-HT seems consistent with the observation by Greenberg et al. (1991) that iloprost does not affect the ability of norepinephrine to contract canine renal artery but relaxes arteries which have been precontracted with the same agent.

While the contraction induced by KCl is very sensitive to the calcium channel antagonist nimodipine, that response is unaffected even by high doses of iloprost. This suggests that the relaxant effect of iloprost in canine cerebral arteries is probably not mediated by effects on voltage-operated calcium channels as suggested by Demirel and Turker (1989) for rabbit vascular preparations. Similarly, the potassium channel opener cromakalim reverses the contraction of canine middle cerebral arteries to low-moderate doses of KCl (Masuzawa et al., 1990), while, as mentioned above, iloprost was devoid of activity against KCl. This suggests that iloprost is not causing relaxation by opening potassium channels.

There is evidence that in a variety of tissues prostacyclin (iloprost) or cAMP derivatives cause elevation of intracellular cyclic AMP and that this is responsible for the relaxation (Murray, 1990). This mechanism would presumably cause relaxation regardless of the agonist responsible for the contraction, and again, the insensitivity of the response to KCl suggests that in canine cerebral arteries, this mechanism may not operate. There are other illustrations of response to KCl which are resistant to cAMP derivatives, for example

bovine coronary artery (Murray 1990). The eicosanoids tested owe their contractile effects to activation of phospholipase C and elevation of inositol (1,4,5)-trisphosphate, with subsequent release of intracellular calcium (Abdel-Latif, 1986). It is possible that iloprost interferes with a step in inositol (1,4,5)-trisphosphate production or possibly with the eicosanoid receptor itself, although there is no evidence in support of the latter suggestion from either binding studies or pharmacological characterization of the iloprost receptor (Dusting and MacDonald, 1990).

Iloprost has been used in an *in vivo* study of cerebral vasospasm in the rabbit, produced either by electrical stimulation or by injection of blood (Egemen et al., 1988), in which the vasoconstriction was reversed by topical application of iloprost. Similar results were reported by Stanworth et al. (1988). Oxyhemoglobin, which is probably the causative factor in the development of cerebral vasospasm, produces cerebrovascular contraction by a mechanism which is not completely understood but which may involve free radicals or eicosanoids (Findlay et al., 1991). Our observations of the ability of iloprost to reverse the short-term effects of oxyhemoglobin and to cause some relaxation of spastic arteries is not only consistent with the in vivo work (Egemen et al., 1988), but in agreement with a similar study by Whalley et al. (1989) in the cat. Further studies of this agent in a model of cerebral vasospasm which approximates more closely to the clinical situation in humans, may be useful.

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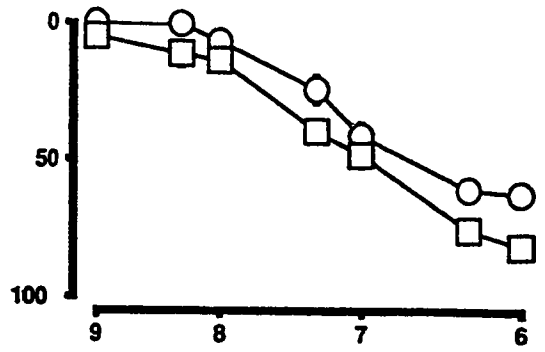

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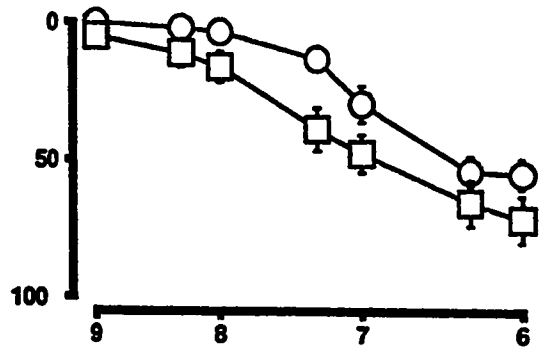

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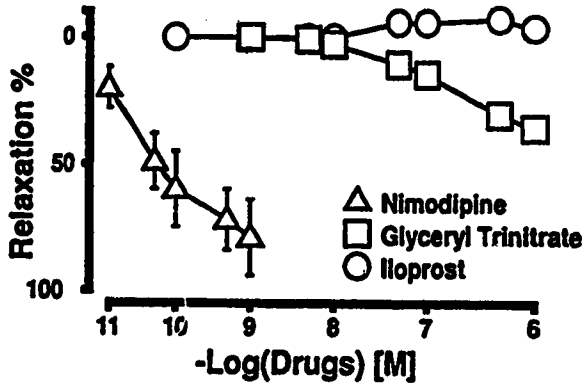

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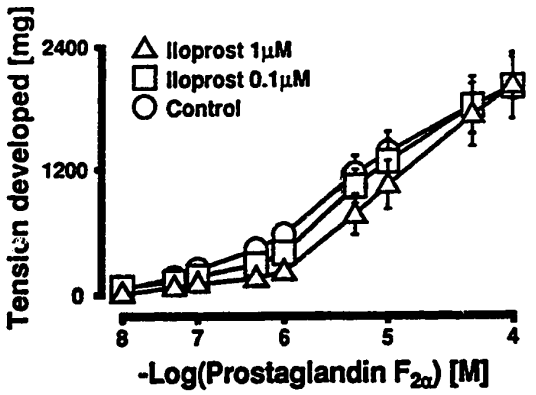
Figure IX-1. Relaxant effects of iloprost (○) and glyceryl trinitrate (□) on the sustained contractions of canine middle cerebral artery induced by (a) prostaglandin $F_{2\alpha}$ (3 μM), (b) U 46619 (1 nM) and (c) KCl (40 mM). In Fig 1c, relaxant effects of nimodipine e (△) are also shown. Bars represent standard errors of the mean for two rings obtained from each of three different animals.



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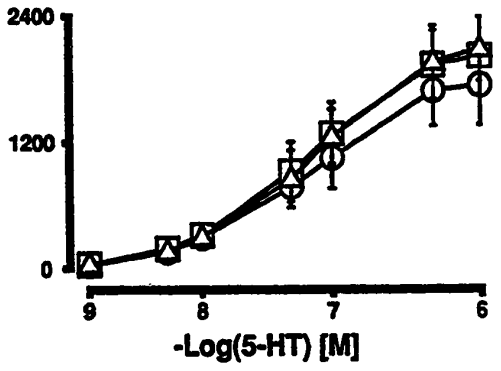

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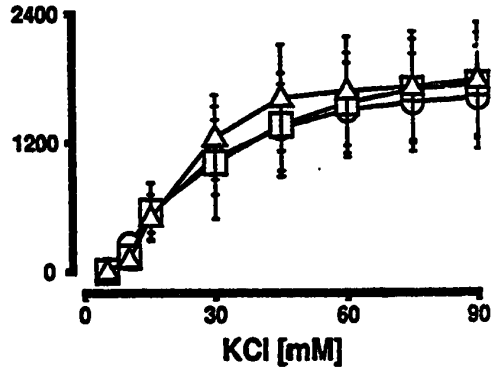

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Figure IX-2. Inhibitory effect of iloprost on the contractile responses of canine middle cerebral artery to prostaglandin $F_{2\alpha}$ (a), 5-HT (b) and KCl (c). Bars represent standard errors of the mean for two rings obtained from each of three different animals. Control (○) data were obtained in the absence of iloprost. Iloprost (0.1 μM (□) or 1 μM (△)) was pre-incubated for 15 min before agonists were administered.

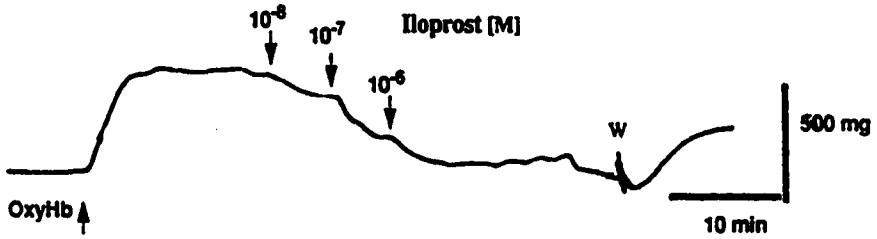

Figure IX-3. Polygraph recording of developed tension against time. Iloprost relaxed the sustained contraction induced by oxyhemoglobin (10 μM) in canine cerebral arteries in a dose-dependent manner. Some contractions were partly recovered after washing.

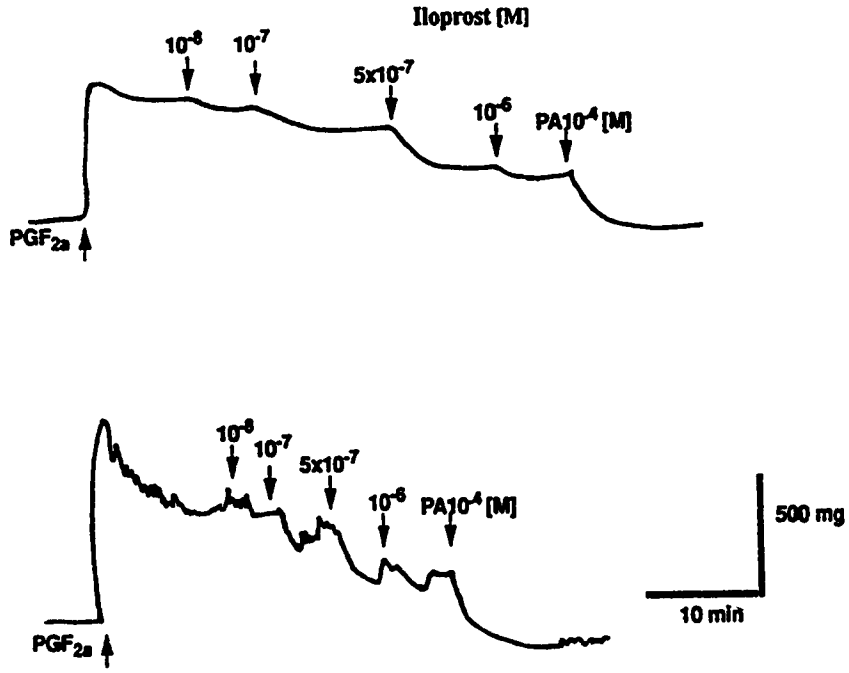

Figure IX-4. Polygraph recording of developed tension against time. Iloprost had similar relaxant effects on the sustained contractions induced by prostaglandin $F_{2\alpha}$ (3 μM) in both control (top) and spastic (bottom) basilar arteries.

CHAPTER TEN

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

SUMMARY AND CONCLUSIONS

As mentioned in chapter two, the membranes of cerebrovascular smooth muscle cells are depolarized during vasospasm after subarachnoid hemorrhage, and this process may play a role in the development and maintenance of the condition. Potassium channel agonists, which shift the membrane potential to a much more negative value than the threshold potential of calcium channels, inhibit the refilling of calcium stores and activate calcium extrusion via sodium-calcium exchange, reduce the intracellular calcium concentration, hyperpolarize the cell membrane and produce muscle relaxation.

In this work, we set out to discover, first, what kind of potassium channels exist in cerebral arterial smooth muscle. In the first and second chapters, the nature of vascular smooth muscle potassium channels and the cerebrovascular effects of some potassium channel modulators were reviewed. Since there have been few studies of cerebrovascular smooth muscle cells using patch-clamp techniques to identify the specific potassium channels, most of the results on potassium channels have previously been obtained from other types of vascular smooth muscle cells. Most of the potassium channels which had been revealed in other tissues, have been identified in vascular smooth muscle, and they seem also to possess similar physiological function. We have identified two distinct potassium channels in rat basilar arterial smooth muscle cells as described in chapter three. The delayed rectifier potassium current is voltage-dependent, shows slow-inactivation, and has slow sigmoidal activation kinetics. This current is reduced by bath application of 1 mM procaine or 1 mM strychnine, but not by 500 nM charybdotoxin. We have noticed, during the preparation of this thesis, that another report (Bonnet et al., 1991) using rat cerebral arterioles had identified a delayed rectifier potassium current which is similar to that which we have observed. Since the repetitive voltage pulses of 1 Hz depressed the peak current, and the reduction of pH increased peak outward current, these authors have claimed that

the cellular mechanism responsible for cerebrovascular dilation to acidosis and/or hypercapnia may involve an increase in outward potassium current (Bonnet et al., 1991). Using higher intracellular calcium concentrations, we have identified a non-inactivating calcium-activated potassium current and a spontaneous transient current, both of which are sensitive to charybdotoxin. Cell-free membrane patches studied with symmetrical potassium concentrations contain 1 or more channels with a conductance of 220 pS with similar voltage and calcium dependence. However, the conductance of the calcium-activated potassium channel is somewhat different from a recent report using rat cerebral arteries (Wang and Mathers, 1992). In their work, a similar voltage- and calcium-dependent potassium channel has been identified but with a smaller conductance of 92 pS. A small inward current, which is activated by hyperpolarizing potentials below -80 to -60 mV has been observed in our study, which is similar to that described by Hirst et al. (1986) from single electrode voltage-clamp recordings of rat middle cerebral arterioles. Since this inward current is small and rarely seen, we have not attempted to characterize it further. We have not observed the classical K_{ATP} channel in our preparation, though it has been shown to exist in vascular smooth muscle (Standen et al., 1989) and to be responsible for the effect of potassium channel modulators (Nelson et al, 1990; Davies et al., 1991). From these studies we conclude that there are at least three distinct potassium channels in cerebral vascular smooth muscle: the delayed rectifier, large conductance or intermediate conductance calcium-activated potassium channels and the inward rectifier. These potassium channels could play a role in membrane stabilization and vessel dilation.

After the potassium channels in rat basilar arterial smooth muscle cell had been characterized, some potassium channel agonists were tested on these currents to explore their mechanism of action. We have shown, in studies reported in chapter four, that cromakalim and pinacidil have no effect on the delayed rectifier potassium current; while at a similar concentration, these drugs activate the calcium-activated potassium channel. The increased current is reduced after the drugs were washed out. In inside-out cell free

patches, cromakalim and pinacidil increase the probability of the opening of the large conductance calcium-activated potassium channel, which has been believed to be the most important component of the whole-cell current. Nicorandil does not affect either component of the whole-cell current, while its effects studied in inside-out single potassium channels were inconsistent. On the basis of these results, we suggest that cromakalim and pinacidil but not nicorandil open calcium-activated potassium channels in rat basilar artery smooth muscle cells. These results are consistent with most patch-clamp studies of potassium channel agonists using vascular smooth muscle (see Table II-1).

We next asked whether those potassium channel agonists which activate the calcium-activated potassium channel in rat basilar arterial smooth muscle cell, could relax canine cerebral arteries. This preparation has been extensively employed in the study of vasospasm. In chapter five, data is presented which shows that nicorandil, pinacidil and lemakalim (the vasorelaxant (-)-trans enantiomer of cromakalim (Edwards and Weston, 1990a)) relax rings of canine cerebral artery precontracted with prostaglandin $F_{2\alpha}$. The order of potency is lemakalim > nicorandil ≅ pinacidil, but all these agents are less effective than nimodipine, a calcium channel antagonist. The effect of nicorandil is inhibited by methylene blue but not by glibenclamide, while the effects of pinacidil and lemakalim are inhibited by glibenclamide but not by methylene blue. Thus, nicorandil probably causes relaxation mostly by effect on guanylate cyclase while lemakalim and pinacidil produce the same effect by action on potassium channels. The results from this pharmacological study are somewhat different from those obtained from patch-clamp studies, but are in agreement with the suggestion of Edwards and Weston (1990a). Since the effects of pinacidil and lemakalim are attenuated by glibenclamide, it might be supposed that the ATP-dependent potassium channel, instead of calcium-activated potassium channel, would be responsible for their action. On the other hand, glibenclamide may block both ATP-dependent- and calcium-activated potassium channels. Since the potassium channels identified in vascular smooth muscle cells are sensitive to either or both ATP and calcium (Gelband et al., 1990),

the nature of the potassium channel with which these agents interact needs further investigation.

During our work on potassium channel agonists, we observed that glibenclamide could *relax* canine cerebral arteries (Zhang et al., 1991a; 1992a). If glibenclamide blocks the action of the potassium channel agonists, as we showed in chapter five (Zhang et al., 1992b; also see Masuzawa et al., 1990), the compound should produce contraction, or at least should not produce relaxation. Thus experiments, described in chapter six, were carried out to examine the mechanism by which glibenclamide relaxes smooth muscle and to explore whether this compound could be useful in the treatment of vasospasm. We have found that glibenclamide can relax the prostaglandin $F_{2\alpha}$-induced contractions in the rat aorta, and in canine femoral, mesenteric, renal, coronary, basilar and middle cerebral arteries. The action of glibenclamide on cerebral arteries is not mediated by cGMP as it is not blocked by methylene blue, an inhibitor of guanylate cyclase, nor is the relaxation endothelium-dependent. Glibenclamide is not only selective for prostaglandin $F_{2\alpha}$, but also blocks contractions induced by prostaglandins E_2 and D_2, and the thromboxane A_2 analog, U46619, while contractions to potassium chloride, noradrenaline, 5-hydroxytryptamine or caffeine are unaffected. The action of glibenclamide is shared by some other sulfonylureas. Glimepiride, glipizide and tolbutamide also have inhibitory effects on the contractile response of rat aorta to prostaglandin $F_{2\alpha}$, while chlorpropamide is devoid of any inhibitory activity.

In addition to its actions on eicosanoids, glibenclamide is surprisingly effective against the contractions of cerebral arteries by oxyhemoglobin (Chapter 7). This is consistant with the hypothesis that at least part of the contractile effect of oxyhemoglobin in cerebral arteries is mediated by eicosanoids. Glibenclamide is thus an agent which selectively blocks the contractile effects of both eicosanoids and oxyhemoglobin. Even though our result is in agreement with another study using canine coronary arteries (Cocks et al, 1990), it is too early to reach any firm conclusion about mechanism, since there are

some reports suggesting that glibenclamide acts as a calcium channel blocker (Chappell et al., 1991; Yoshitake et al., 1991). While this effect would account for the relaxation to eicosanoids, it would be anticipated that responses to potassium chloride would be very sensitive to glibenclamide, which is not what we observed. It is possible that this agent has a multitude of effects depending on the preparation studied and possibly on other circumstances.

The possible use of those potassium channel agonists in management of cerebral vasospasm has been investigated using rings of canine cerebral artery. Preliminary experiments (not discussed in this thesis) using nicorandil, pinacidil and lemakalim revealed that these compounds had showed some degree of relaxant effect against the contraction induced by oxyhemoglobin. In order to determined the cerebrovascular effect of endogenous potassium channel openers, reviewed in chapter two, we have studied the action of iloprost, which is an analog of prostacyclin and has been shown to relax vascular smooth muscles by an increase intracellular cAMP (Murray, 1990) and/or by opening of potassium channels (Siegel et al., 1989). We have tested the effect of iloprost in canine cerebral arteries against eicosanoids and oxyhemoglobin, and in spastic or normal arteries. Iloprost causes relaxation of canine cerebral arteries precontracted with oxyhemoglobin, prostaglandin $F_{2\alpha}$ or the thromboxane A_2 analogue, U 46619. The presence of a moderate delayed vasospasm in the tested arteries provides similar result to that obtained in control arteries. Since oxyhemoglobin is believed the most probable etiological reason for vasospasm, it is possible that iloprost, as well as those synthetic potassium channel agonists, could play a role in the treatment of vasospasm.

RECOMMENDATIONS

ION CHANNEL STUDIES.

Cerebrovascular Potassium Channels. Three distinct potassium channels in cerebral vessels have been identified. These are the delayed rectifiers (Zhang et al., 1991b; Stockbridge et al., 1992; Bonnet et al., 1991), large (Zhang et al., 1991b; Stockbridge et al., 1992) and intermediate (Wang and Mathers, 1991) conductance calcium-activated potassium channels and inward potassium currents (Edwards et al., 1988; Zhang et al., 1991b). In addition to these voltage-dependent or calcium-activated potassium channels, there are many other types of potassium channels which are known to exist in vascular smooth muscle, as described in chapter two, especially potassium channels regulated by G-protein-coupled second messenger systems or by phosphorylation. These potassium channels are directly or indirectly coupled to their associated receptors and probably play an active role in the control of muscle tone. The possible existence and function of other potassium channels in cerebrovascular smooth muscle is an important area which needs further study.

With regard to potassium channel agonists, a lot of work has been done using the patch-clamp technique, but we are still far from a convincing conclusion as to what type (s) of potassium channels is responsible for their action in blood vessels. Most authorities claim that the ATP-dependent potassium channel is activated by potassium channel openers in cardiac myocytes, while the calcium-activated potassium channel, ATP-dependent or delayed rectifier potassium channel is activated in vascular smooth muscle (Table II-1). Further work needs to be done to test these potassium channels in the same tissue (Tseng et al., 1990) and at the same temperature (Sanguinetti et al., 1988).

Glibenclamide is generally believed to block the ATP-dependent potassium channel, and thus to antagonize the actions of such drugs as pinacidil or cromakalim. While it interferes with these agents in cerebrovascular smooth muscle cells, it is probable that the

pinacidil-like drugs are interfering with the important calcium-activated potassium conductance rather than K_{ATP}, whose presence we have not convincingly demonstrated. When these observations are added to the reports that the general potassium channel blockers, such as 4-AP, TEA and procaine are non-specific antagonists of the actions of potassium channel openers (Hamilton & Weston, 1989), and the actions of apamin and charybdotoxin, which selectively block small and large conductance calcium-activated potassium channels are not sensitive to potassium channel openers (Winquist et al., 1989), it is clear that this highly confusing situation needs to be resolved.

Another area of interest is the search for drugs specific to the cerebral vasculature. The known potassium channel agonists, such as nicorandil, pinacidil and cromakalim are effective in coronary or peripheral arteries and have been used in management of both angina pectoris and hypertension. In view of the fast expansion of our knowledge of potassium channel agonist families (Edwards and Weston, 1990b), it is possible that a more specific drug for cerebral arteries could exist. Since the action of potassium channel agonists becomes more significant under ischemic conditions, and since decreased membrane potassium conductance may be a major contributory factor in cerebral vasospasm, such a cerebrovascular selective potassium channel agonist would show great promise in the management of cerebral vasospasm (Duty and Weston, 1990). The search for such a drug is an important and exciting project.

Cerebrovascular Calcium Channels. If potassium channels play a major role in the regulation of vessel dilatation, calcium channels are known to play a significant role in the vessel constriction (Nelson et al., 1990). Two calcium currents have been observed in preliminary experiments in this laboratory, which resemble the L- and T-type of calcium currents identified in other tissues. Since the pathophysiological change of cerebral vasospasm is prolonged constriction of blood vessels (Findlay et al., 1991), and there is some evidence that calcium channel antagonists may reverse angiographic vasospasm (Flamm et al., 1988; Shibuya et al., 1988), the study of calcium channels and their

antagonism in the cerebral vasculature using the patch-clamp method would be interesting. Also, the study of the possible changes of calcium current upon the application of oxyhemoglobin will benefit our understanding of the pathophysiology of vasospasm.

IN VIVO STUDY OF POTASSIUM CHANNEL MODULATORS

Synthetic Potassium Channel Agonists. Nicorandil has been reported to hyperpolarize smooth muscle, increase the internal diameter, and reverse the reduction in basilar artery diameter after experimental subarachnoid hemorrhage (Harder et al., 1987). This finding has been confirmed by several other studies (Yamada et al., 1989; Sakakura and Wage, 1990). Pinacidil relaxes cerebrovascular smooth muscle with a potency comparable to the calcium channel antagonists (Toda et al., 1985; Wahl, 1989; Zhang et al., 1992b). Cromakalim shows larger effects on cerebral arteries than peripheral arteries (Grant and O'Hara, 1990). These potassium channel agonists have been tested clinically for the treatment of hypertension, angina pectoris, and experimentally for asthma and peripheral vascular disease (Hamilton and Weston, 1989). Application of these potassium channel agonists to animal model of cerebral vasospasm perhaps leading to clinical trials should be encouraged.

Endogenous Potassium Channel Agonists. As described in chapter two, a variety of endogenous potassium channel agonists exist, and also possess other actions on the vessel. Prostacyclin, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) increase cytosolic cAMP, possibly activate the ATP-dependent potassium channel (Standen et al., 1989), hyperpolarize the membrane and thus relax cerebral smooth muscle. Prostacyclin or its analog iloprost, relaxes spastic arteries (Zhang et al., 1992c), reverses vasospasm in models in the cat or dog (Egemen et al., 1988; 1990) and improves the clinical condition of patients in vasospasm (Stanworth et al., 1988). Substance P (SP), atrial natriuretic peptide (ANP), adenosine and nitrovasodilators increase cytosolic cGMP and open potassium channels, possibly the calcium-activated potassium channel (Williams et al., 1988; Fujino et al., 1991). The effect of nitrovasodilators in

vasospasm has been studied in both experimental models and in the clinical situation. Continuous intravenous infusion of nitroglycerin for 14 days in patients with ruptured cerebral aneurysms prevented severe vasospasm and improved the surgical results (Goto et al., 1990). Needless to say, more *in vivo* studies or clinical trials are called for, to test the effect of endogenous potassium channel agonists with properties similar to nitroglycerin. The complex activities of these endogenous compounds can proceed via a variety of mechanisms including release of other endogenous vasodilators, actions on membrane ion channels and effects on a variety of second messengers including cAMP, cGMP and conceivably inositol 1,4,5 trisphosphate or diacylglycerol. Detailed examination of their mechanism of action is important and may suggest new approaches not only to management of cerebral vasospasm, but to other conditions affecting the contractility of blood vessel.

POTASSIUM CHANNEL ANTAGONIST-SULFONYLUREAS

Although glibenclamide and other sulfonylureas depolarize vascular smooth muscle (McPherson and Angus, 1991) and block the vasorelaxant effect of potassium channel agonists (Buckingham et al., 1989; Cavero et al., 1989; Zhang et al., 1992b), unlike most potassium channel antagonists, these agents *relax* smooth muscle contractions induced by eicosanoids and oxyhemoglobin (Cocks et al., 1990; Zhang et al., 1991a; 1992a) and possibly other agents (Chappell et al., 1991; Yoshitake et al., 1991). The mechanisms which underlie their ability to cause relaxation is unclear. One possibility is the specific inhibition of eicosanoids as suggested by Cocks et al. (1990) and by our experiments. This suggestion could be confirmed by a binding study using eicosanoid receptors. Another, although less probable hypothesis (Schmid-Antomarchi et al., 1987), is that these agents owe their effects to blockade of calcium channels (Chappell et al., 1991). A further suggestion is that glibenclamide blocks ATP-dependent potassium channels at lower concentrations, but activates potassium channels at higher doses (Hu et al., personal communication). All these ideas need further study. Finally, the clinical effect of the

sulfonylureas seems closely and positively related to their potencies in blocking the potassium channel (Schmid-Antomarchi et al., 1987), as well as their inhibition against prostaglandin $F_{2\alpha}$ (Zhang et al., unpublished observation). It remains to be seen whether the vasorelaxant action can be exploited clinically in patients with cerebral vasospasm.

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