INVESTIGATION OF THE EFFECT OF SOME POTASSIUM CHANNEL BLOCKERS ON NEURONAL AND ENDOTHELIAL MODULATIONS OF SMOOTH MUSCLE TONE IN DIFFERENT TYPES OF BLOOD VESSELS

Summary of PhD thesis

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- 2003 -

INTRODUCTION

Coordination and integration of cardiac and peripheral vascular activities are essential for the maintenance of homeostasis in the organism. The peripheral circulation plays an essential role in this interplay and can be classified in different categories: conduit, capacitance and resistance blood vessels. The arteries, as *conduits*, have the role of carrying an adequate supply of blood from the heart to the peripheral organs and tissues of the body. The efficiency of the arterial conduit function depends on the arterial caliber and the constancy of mean blood pressure. *Capacitance vessels* (mainly the venous section) can take in or pass on large volumes of blood with no marked effects on the other parameters of the circulation. They can thus act as blood reservoirs. Changes of smooth muscle tone in these vessels can produce hemodynamically important shifts in regional blood content and thus influence venous return and cardiac output. *Resistance vessels* determine the overall resistance function, and hence, blood flow. The greatest resistance to flow is in the precapillary region (terminal arteries and arterioles). Activity of the smooth muscles in these arteries is the decisive factor in the regulation of volume flow within each vascular bed, as well as in the distribution of the cardiac output among the various organs. The postcapillary resistance is determined by the venules (and veins).

Vascular tone, the contractile activity of vascular smooth muscle cells, is the major determinant in the regulation of blood pressure and tissue perfusion. The contractile activity depends on complex interplay of different vasoconstrictor and vasodilator stimuli. These factors, which determine vascular tone and hence the diameter of blood vessel, are derived from neurons, endothelium or generated by smooth muscle cells. One of the major factors affecting the vascular tone is the membrane potential. Membrane potential of neurons, endothelial and smooth muscle cells are regulated by a variety of voltage- and ligand-gated potassium channels.

Smooth muscle cells are the most intensively investigated vascular cells. Membrane potential of smooth muscle cells appears to be an important regulator of vascular tone. Vascular smooth muscle cells express 4 different types of K^+ channels: voltage-dependent K^+ (K_V) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, ATP-sensitive K^+ (K_{ATP}) channels and inward rectifier K^+ (K_{IR}) channels. The opening of K^+ channels in the membrane of smooth muscle cells increases K^+ efflux out of the cells leading to membrane hyperpolarization. Closure of K^+ channels has the opposite effect. Hyperpolarization closes the voltage-gated Ca^{2+} channels, which causes vasodilation, whereas depolarization opens them, inducing vasoconstriction.

The vascular *endothelium* is located at the interface between the circulating blood and vessel wall. Endothelial cells, unlike nerve and smooth muscle cells, are classified as nonexcitable cells since they have never been observed to produce action potentials. The endothelium interacts with smooth muscle cells by the synthesis and secretion of vasoactive mediators and hence modulates the vascular tone and cardiovascular homeostasis. The most widely distributed ion channels in endothelial cells are K^+ channels. There are at least four types of endothelial K^+ channels: 4-AP sensitive K^+ channels, Ca^{2+}-activated K^+ channels, ATP-sensitive K^+ channels and inward rectifier K^+ channels. The K^+ channels play a role in maintaining the resting membrane potential and the regulation of intracellular free Ca^{2+} concentration following stimulation by neurohumoral and physiological stimuli. Recently the role of K^+ channels has been investigated in the release of vasoactive substances suggesting activation of endothelial K^+ channels in the release of NO.

Blood vessels are innervated by sympathetic nerves. The release of different transmitters from *presynaptic nerve* terminal depends on the electrical responses of presynaptic membrane. K^+ channels are important participants of electrical changes in membrane potential leading to transmitter release. The presynaptic nerve terminals also contain the four main types of K^+ channels as described in smooth muscle or endothelial cells.

AIMS OF THE THESIS

In the light of these limited informations, K_V and K_{Ca} channels seem to be the most important K^+ channels in the regulation of smooth muscle cells, endothelial cells or perivascular nerves. The main purpose of the present study was to identify the functional role of K_V and K_{Ca} channels in the regulation of blood vessels by applying some potassium channel blockers. The aims were subdivided as follows:

- Investigation of the role of potassium channels on conduit, capacitance and resistance types of blood vessels.
- Investigation of the role of K_V channels in the regulation of transmitter release.
- Investigation of the role of endothelium in the regulation of transmitter release and a modulatory role of K_V channels in endothelium.
- Investigation of the effect of K_V channel blockers on basal vascular tone.
- Investigation of the role of K_{Ca} channels in the regulation of transmitter release.

METHODS

Tissue preparation and mounting of conduit blood vessels

Epicardial coronary arteries obtained from porcine hearts were cleaned from the adhering connective tissue and the blood vessels were cut into 5 mm ring segments. Rings were placed into an organ bath filled with Krebs-Henseleit Solution (KHS) and were aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C. The isometric tension was recorded with a force-displacement transducer. Strips were stretched passively up to 30 mN and equilibrated for 45 min, during which the medium was changed every 15 min.

Human coronary arteries were prepared from parts of undiseased donor hearts unsuitable for transplantation from which the aortic and pulmonary valves had been previously excised for homograft-valve surgery. Before explantation of the hearts the patients did not recieve any medication except for dobutamine, furosemide and plasma expanders. The experimental protocol complied with the Declaration of World Medical Association proclaimed in Helsinki and was approved by the Human Ethical Review Board of the Albert Szent-Györgyi Medical University (No. 51-57/1997 OEj). The hearts were stored in cardioplegic solution at 4°C and used for the experiments within 12 hours. The human coronary preparation was stretched up to 10 mN instead of the 30 mN.

The *thoracic aorta* of New-Zealand white rabbits was cut into 5 mm ring segments for isometric tension recordings. Rings were placed into 4 ml KHS and were stretched passively to 30 mN and equilibrated for 45 min.

Tissue preparation and mounting of capacitance blood vessels

Lateral saphenous veins of mongrel dogs were carefully cleaned from connective tissue and were cut into 5 or 30 mm ring segments for investigation of the tone or for radioactive studies, respectively. The 5 mm ring segments were stretched passively to 10 mN and equilibrated in 2 ml KHS for 45 min.

Human portal vein preparations were obtained from general organ donor patients whose liver was undergoing transplantation surgery. The permission, the preparation and the handling of portal veins, was the same as described above, i.e. as in the case of human coronary arteries. The venous preparation was stretched up to 10 mN during the equilibration period.

Tissue preparation and mounting of resistance blood vessels

The *penile small artery* was carefully dissected from the rat corpus cavernosum and cleaned from the adherent connective tissue. Two mm long arterial rings were cut and mounted on two 40 μm wires in an isometric double-myograph. Before starting the experiment, vessels were allowed to equilibrate in physiological salt solution for about 30 min. The relation between resting wall tension and internal circumference of the vessels was set, and the internal circumference, $L_{100,}$ corresponding to a transmural pressure of 100 mmHg in a relaxed vessel *in situ*, was calculated. Subsequently, the internal circumference of the vessels was set to L_1, where $L_{1=}0.9 \times L_{100}$.

Denudation process and verification of endothelial function

Removal of the endothelium of different blood vessels was achieved mechanically by using a wet cotton swab on a glass rod except for rat penile small arteries where the endothelium removing was performed by bubbling of air. Functional endothelial denudation was evidenced by lacking vascular relaxation after administration of 1 μM bradykinin or by exposure to 10 μM acetylcholine.

Loading procedure in conduit and capacitive blood vessels

Loading procedure of the preparations with noradrenaline (NA) or 5-hydroxytryptamine (5-HT) involved the addition and incubation of the preparations with 1 mM NA or 1 mM 5-HT for 10 - 30 min followed by thorough washing out (at least 3 times) of the amine from the organ bath. After washing out this large concentration of NA or 5-HT the changes of tone were monitored again by repeated exposure to the applied potassium channel blocking agents.

Experiments with ^{3}H-noradrenaline in canine saphenous vein

The 30 mm ring segments were equilibrated and incubated with ^{3}H-noradrenaline (3.8 nM) and unlabelled NA (1 μM). After loading with ^{3}H-NA the venous rings were washed and further incubated for 20 min in 2 ml KHS. At the end of the experiments the rings were washed again one time and the total incubation medium (4 ml) and the ring vessels were collected for estimation of radioactivity. Experiments were repeated in the presence of 1.25 and 5 μM 4-aminopyridine (4-AP).

Electrical field stimulations in rat penile small arteries

Electrical field stimulation (EFS) was performed through two parallel platinum electrodes placed in parallel with the vessel segment approximately 2 mm apart from each other. The preparations were stimulated transmurally by trains with 0.3 ms square pulses applied at frequencies of 1-32 Hz for 20 sec. The current output of the stimulator was constant and adjusted to 40 mA.

RESULTS AND DISCUSSION

Investigation of the effect of potassium channel blockers on conduit blood vessel preparations

The results of the present investigations demonstrate that the K_V channel blocker, 4-aminopyridine (4-AP), and the K_{Ca} channel blocker, tetraethylammonium (TEA), induce contractions of porcine and human coronary arteries via the release of vasoconstrictor 5-HT. After loading of coronary arteries with 1 mM 5-HT, 4-AP induced significantly larger contraction and methysergide abolished this contraction both in porcine and in human preparations. This suggested an intact 5-HT storage capacity and intact uptake mechanism of coronary arteries under our experimental conditions. Based on the present findings we propose that under pathological conditions the neuronal stores of coronary arteries are filled with platelet-derived 5-HT. This serotonin may subsequently contribute to an abnormal contractile responsiveness, i.e. vasospasm of the coronary artery. The 5-HT stores of the blood vessel wall may be strongly affected by drugs acting on K_V -type potassium channels and, conseqently influence the coronary tone. The current findings suggest that 4-AP can induce spasm of the coronary artery with consequent ischemic damage of heart muscle. This harmful effect of the K_V channel inhibitors may have *in vivo* significance when the neuronal stores contain large concentration of 5-HT.

In our investigations, 4-AP induced dose-dependent contraction in rabbit aortic preparations. This enhancement of basal tone was sensitive to the α-receptor blocker phentolamine supporting the involvement of NA release in the contractile effect of the potassium channel blocker. This is in agreement with our observations made in porcine and human coronary arteries that the K_V channel located on the neuronal membrane plays an important role in the regulation of blood vessel tone by releasing a vasoactive neurotransmitter. Earlier observations on isolated rabbit ear arteries, on cat cerebral and femoral arteries and on rabbit pulmonary artery and aorta also confirm this finding. Moreover, removal of endothelium in rabbit aortic preparations failed to modulate either the maximum contractile effect or pD_2 values of the K_V channel blocker in our experiments.

In porcine coronary artery, with intact endothelium, we characterized a nitric oxide (NO) and an endothelium-derived hyperpolarization factor (EDHF) mediated relaxation induced by bradykinin by using known endothelial inhibitors: the NO synthase inhibitor L-NOARG, the cyclooxygenase enzyme inhibitor indomethacin (INDO) and TEA. In the presence of L-NOARG, the BK induced relaxation was significantly decreased suggesting the release of NO from endothelium. The BK-induced relaxation is TEA-sensitive, since the K_{Ca} channel blocker decreased the relaxation evoked by BK. TEA also decreased the persistent relaxation in the presence of L-NOARG plus INDO, suggesting the presence of K_{Ca} channel sensitive release of a non-NO non-prostanoid factor, which corresponds to EDHF. In porcine coronary artery the presence of such a functional EDHF and its modulation by K_{Ca} channel has been suggested recently. Activation of K^+ channels hyperpolarizes endothelial cell and thereby supports Ca^{2+} influx resulting in production and release of NO and EDHF. In contrast, depolarization of endothelial cells with K^+ channel blockers decreases the Ca^{2+} signal and reduces the release of vasoactive substances.

Investigation of the effect of potassium channel blockers on capacitance blood vessel preparations

The main finding of our present study is that even submicromolar concentrations of 4-AP are able to enhance the basal tone of canine isolated saphenous vein. The calculated EC_{50} values of the drug are 0.61 μM and 0.53 μM in the venous rings without and with endothelium, respectively, when the venous neuronal stores are loaded with noradrenaline. These values are almost the same as the serum level measured in multiple sclerosis. The observed high potency in our present study supports our previous assumption that 4-AP may have contractile effects on the smooth muscles in the circulatory system. It is important to note that this was evident under those experimental conditions where the venous tissue was loaded with noradrenaline (NA). Loading the saphenous preparations with large concentrations of NA resulted in an increased efficacy and potency of 4-AP when compared to the responsiveness of the unloaded veins. The maximum contractile effect of 4-AP increased 5-8 fold when the perivascular neuronal stores were filled with NA.

Because of the relatively intact storage capacity of the perivascular nerve endings in the blood vessel following isolation procedures, and also the striking sensitivity of the nerves to the effect of 4-AP, we measured the influence of chemical denervation in a saphenous preparation. After loading the NA store of the venous tissue, 6-hydroxydopamine completely abolished the contractile effect up to the largest concentration of 4-AP applied (5 μM). This finding suggests an indirect neuronal mediation of the effect of low concentrations of 4-AP on venous tone.

In order to detect the involvement of NA as contractile substance in the mechanism of action of low 4-AP concentrations, determination of the release of radiolabelled NA was performed. 5 μM 4-AP significantly increased the tritium efflux from the venous tissue compared to the control fractional efflux of untreated

preparations. This shows that in isolated canine saphenous vein the contractile effect of 4-AP is largely dependent on the neural release of NA.

Although 4-AP sensitive K_V channels have been shown to be present in the membrane of endothelial cells of bovine aorta and pulmonary artery, is no evidence for the existence of a 4-AP sensitive K^+ channels in the endothelium of canine saphenous vein has been presented. Hence, together with the observation that chemical denervation completely inhibited the 4-AP induced contractions, the lack of influence of vascular endothelium also points to the predominant role of a neuronal mechanism in the effect of this K_V channel blocker.

4-AP induced dose-dependent contraction in human portal vein preparation, indicating the presence of K_V channel in this blood vessel. This is in agreement with earlier findings, supporting that K_V channels play a crucial role in the regulation of the blood vessel tone in portal venous preparations obtained from different species. Although we have not yet characterized the exact mechanism of contraction evoked by 4-AP in human portal vein, we suppose the release of vasoconstrictor neurotransmitter(s) in the contractile effect of 4-AP, since the lowest concentration used in the experiment induced almost maximal contraction. This finding correlates with low micromolar pD_2 values observed in canine saphenous vein.

In summary, it appears that the veins are the most sensitive *in vitro* preparations to the contractile effect of 4-AP. The effect of the submicromolar concentrations of the K_V channel blocker and the involvement of NA in the mechanism of action on canine saphenous vein as well as the large sensitivity of human portal vein to 4-AP support the *in vivo* significance of the findings.

Investigation of the effect of potassium channel blockers on resistance blood vessel preparations

4-AP, blocker of K_V channels, and TEA, iberiotoxin (IbTX) as well as charybdotoxin (ChTX), blockers of K_{Ca} channels significantly increased the basal tension of rat penile small arteries in the presence of endothelium. Although, the role of potassium channels in the regulation of basal tension of different blood vessels has been previously demonstrated, such comparative experiments with the K^+ channel blockers in penile arteries have not yet been performed. Moreover, in our experiments these contractions are not affected by the presence of guanethidine, atropine and L-NOARG and these findings exclude the involvement of K^+ channel modulation by adrenergic, cholinergic and nitrergic nerves. On the basis of these findings, K_V and large-conductance K_{Ca} channels appear to be major regulators of the basal tone of rat penile small arteries.

In rat penile small arteries EFS produced frequency-dependent contractions at baseline tension, which was abolished by guanethidine or prazosine treatment. This finding suggested the neuronal origin of EFS evoked contractions. The blockers of Ca^{2+}-activated K^+ channels, tetraethylammonium and charybdotoxin markedly enhanced EFS-evoked contraction. In contrast to this, 4-aminopyridine and glibenclamide did not modulate contractions induced by EFS suggesting that voltage-dependent K^+ channels and ATP-sensitive K^+ channels are not involved in these responses.

Endothelial cells have been described either to mediate, to inhibit or not to play any role in the neurogenic responses elicited by EFS in arterial preparations. In the present study removal of the endothelial cell layer also increased EFS and noradrenaline-evoked contraction. In several preparations the inhibitory effect of the endothelial cell layer on neurogenic responses has been ascribed to release of NO. However, in the present study an inhibitor of NO synthase, L-NOARG, was included in all experiments excluding the contribution of NO. Moreover, an inhibitor of cyclooxygenase, indomethacin, did not enhance the neurogenic contractions. Therefore, inhibition of the neurogenic contractions can probably be ascribed to a non-NO non-prostanoid endothelium-derived factor.

In penile small arteries acetylcholine and bradykinin causes EDHF type relaxation which persists in the presence of inhibitors of cyclooxygenase and NOS enzymes. In horse penile arteries a combination of small- and intermediate K_{Ca} channel blockers, apamin and ChTX, caused inhibition of EDHF-type relaxation evoked by acetylcholine and bradykinin. In the present study TEA blocked the EDHF-type relaxation and, surprisingly, incubation with ChTX alone was sufficient to abolish this EDHF-type relaxation evoked by acetylcholine. These observations suggest the role of intermediate-conductance K_{Ca} channels in EDHF-type relaxing responses evoked by acetylcholine in rat penile arteries.

In contrast to the endothelium intact preparations, in endothelium-denuded segments TEA did not increase EFS-evoked contractions at high frequency stimulation (4-32 Hz). In rat penile arteries only a blocker of intermediate-conductance K_{Ca} channels, ChTX, increased the neurogenic contractions in endothelium-intact preparations. In contrast, apamin and IbTX that block small- and large-conductance K_{Ca} channels, respectively, did not change the neurogenic contractions. Therefore, our results reveal intermediate-conductance K_{Ca} channels sensitive to TEA and ChTX, which are localized in the endothelial cell layer, and are involved in the inhibition of the release of vasoconstrictor neurotransmitter. However, in endothelium-denuded preparations TEA increased EFS-evoked

contractions at low frequency stimulation (1-2 Hz) which suggest the role of sympathetic nerve endings in particular conditions.

Experiments of neurogenic relaxations were performed in the presence of guanethidine and atropine to obtain non-adrenergic non-cholinergic conditions. In our study, the inhibition of NO synthesis by L-NOARG reduced the EFS induced relaxations, indicating the involvement of NO in the relaxation in the present study.

The non-selective K_{Ca} channel blocker, TEA, and the K_V channel blocker, 4-AP, but not the K_{ATP} channel blocker, glibenclamide, attenuated the inhibitory relaxation evoked by EFS. This effect of TEA and 4-AP was not likely to be due to prejunctional modulation of the release of nitrergic transmitter, since the vasodilation induced by exogenously added NO, as acidified $NaNO_2$, was also reduced by the blockers. The involvement of K_{Ca} and K_V channels in NO-mediated relaxations has recently been demonstrated in different types of arteries. In contrast, the K_{ATP} channel blocker glibenclamide failed to exert any modulatory effect on the relaxation induced by either EFS or NO as acidified $NaNO_2$. Our observation provides evidence for activation of K_{Ca} and K_V, but not K_{ATP} channel by NO in rat small penile arteries.

SUMMARY

The main findings of the present thesis are as follows:

1. The K_V and K_{Ca} channels play a crucial role in setting the resting vasomotor tone of conduit blood vessels via the regulation of the release of vasoconstrictor mediators, 5-hydroxytryptamine or noradrenaline. The pharmacodynamic effect of 4-AP in the human coronary artery resembles that observed in the porcine coronary artery. This suggests that coronary artery preparations obtained from the porcine heart can serve as a model for studying the functional effect of drugs on K_V and K_{Ca}-types of potassium channels.
2. Even submicromolar concentrations of 4-aminopyridine are able to increase the tone of canine saphenous vein under experimental conditions. We could demonstrate the role of noradrenaline in the mediation of the effect of 4-AP, which suggests the involvement of an indirect neuronal mechanism in the effect of this K_V channel blocker. From these results it seems fair to conclude that the actual sympathetic activity profoundly affects the contractile efficiency of 4-AP in veins.
3. In rat small penile arteries K_V and K_{Ca} channels play an important role in the regulation of smooth muscle and endothelial cells. Electrical field stimulation induced contractions are modulated by a non-nitric oxide non-prostanoid relaxing factor released from endothelium through activation of ChTX-sensitive K_{Ca} channels located on the membrane of endothelial cells. NO and unknown relaxing factor(s) released from non-adrenergic non-cholinergic nerves through electrical field stimulation modulate the K_{Ca} and K_V channels located on smooth muscle cells. The relaxing factors, released from either endothelium or non-adrenergic non-cholinergic nerve endings, may participate in the physiological regulation of erection leading to relaxation of arteries and trabecular smooth muscle. Thus, application of Ca^{2+}-activated K^+ channel openers would provide a potential pharmacological treatment for erectile dysfunction.
4. EDHF is an important vasodilator agent in porcine coronary and rat penile small arteries. The K_{Ca} channel participates in the vasodilator mechanism of EDHF in these arteries.
5. Presence or absence of endothelium does not modulate the contractile effect of the K_V channel blocker, 4-AP on conduit and capacitance blood vessel preparations *in vitro*, suggesting the efficacy of this agent in the presence of pathologically damaged endothelium of both conduit and capacitance types of blood vessels.

LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

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V. **Kun A**, Martinez AC, Tankó L, Pataricza J, Papp JGy, Simonsen U (2003) Ca^{2+}-activated K^{+} channels in the endothelial cell layer involved in modulation of neurogenic contractions in rat penile arteries.
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ACKNOWLEDGEMENTS

I am very grateful to Professor **Julius Gy. Papp**, MD, DSc, academian, for his continuous support and for providing me the opportunity for research as PhD student at the Department of Pharmacology and Pharmacotherapy, University of Szeged and later as research assistant at the Division of Cardiovascular Pharmacology, Hungarian Academy of Sciences. His inspirational comments and constructive criticism were always of help and are greatly appreciated.

I am especially thankful to my PhD supervisor **János Pataricza**, MD, PhD, for personal guidance and for introducing me to the fascinating world of experimental pharmacology. I always enjoyed his optimistic attitude to the scientific problems. Without his continuous support, never-failing interest and eagerness to discuss new ideas, plans and findings throughout these years, this PhD study could have hardly come to an end.

Ulf Simonsen MD, PhD, is sincerely thanked for excellent collaboration during the years and for the many hours of splendid and witty scientific discussions that gave me lot of ”never-to-forget” lessons.

László Tankó MD, PhD, and **Ana Cristina Martinez** PhD, are also gratefully acknowledged for inspiring discussions and lots of excellent advices.

Zsuzsanna Süli is also greatly appreciated for her technical assistance and for creating a cheerful and social milieu in the laboratory.

I also wish to thank my wife (**Diána Szabó** MD, PhD) and my mother (**Katalin Bene**) for their love, patience and trust.

Last but not least, all colleagues and staff members at the Department of Pharmacology and Pharmacotherapy are gratefully thanked for creating a supportive and pleasant working environment.

This work was supported by grants from the Hungarian Ministry of Health (ETT T-144/2001, 03 293/01), Hungarian National Research Foundation (OTKA T-12848, T-030301, T-037520) and from the Hungarian Academy of Sciences.