EFFECT OF CAPSAICIN ON THE FUNCTION OF ISOLATED HEART UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS. POSSIBLE ROLE OF NEURAL ENDOTHELIN

Ph.D.-értekezés

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Content

General introduction

Our knowledge concerning the local role of the capsaicin-sensitive primary afferent neurones in various organs and tissues has immensely expanded over the past decades. Capsaicin-sensitive nerve endings are involved in local effector function in a wide variety of organs (Szolcsanyi 1984), beyond conveying information from the periphery to the central nervous system according to the classical Cartesian reflex principle. The functional significance of this unorthodox dual sensory-effector function has been increasingly recognised, although the effect of capsaicin on the heart has not been studied in detail (Maggi 1995, Lundberg 1996).

Antidromic stimulation of dorsal roots or peripheral axons of somatosensory neurones in the skin evokes: (1) arterial vasodilatation (Bayliss, 1901; Celander and Folkow, 1953; enhanced microcirculation (Szolcsanyi, 1988) or flare reaction, (2) venular plasma extravasation (Jancso et al., 1967, Szolcsanyi 1984), the latter underlying a pivotal early step in inflammation. The neurotransmitters responsible for the neurogenic inflammation include substance P and neurokinin A mediating plasma extravasation, and calcitonin gene-related peptide (CGRP) accounting for vasodilatation (Szolcsanyi 1984, Maggi 1995, Lundberg 1993). Neurogenic inflammation participates not only in acute but also in a range of chronic inflammatory processes such as rheumatoid arthritis, conjunctivitis, psoriatic skin disease and bronchial asthma (Geppetti and Holzer 1996).

With regard to the respiratory system, atropine-resistant neurogenic tracheobronchial response provoked by capsaicin or electrical stimulation of the nerve fibres was demonstrated in rats and guinea pigs (Szolcsanyi and Bartho 1982; Lundberg and Saria 1982). Subsequently, the mediator role of neurokinin A and substance P was suggested.

With respect to the gastrointestinal tract, capsaicin and other stimulants of gastric and intestinal capsaicin sensitive afferents enhance mucosal blood flow. providing protection against various experimental damage to gastric and intestinal mucosa in rat and rabbit (Szolcsanyi and Bartho 1981, Holzer 1992, Abdel-Salam et al., 1994). In fact the first evidence for a smooth muscle response in an isolated tissue

in response to excitation of capsaicin-sensitive fibres was obtained with guinea-pig isolated ileum (Szolcsanyi and Bartho 1978, Bartho and Szolcsanyi 1978).

In the genitourinary system, excitation of peripheral terminals of capsaicin-sensitive primary afferents promotes the pyeloureteral propulsion motility mediated by tachykinin release (Maggi 1995). The local efferent function of sensory nerve endings has also been characterised in a wide range of other organ systems including the eye, skeletal muscle, joints and bone and even in the lymphoid organs and the immune system (Maggi 1995).

During the past years, novel facets of the dual function of capsaicin-sensitive sensory nerve terminals have also been clarified. Besides the local efferent functions mentioned above a systemic anti-inflammatory impact has been demonstrated (Szolcsanyi et al., 1998a,b). In response to both antidromic and orthodromic excitation of capsaicin-sensitive afferents beyond local neurogenic inflammation an anti-inflammatory effect developed at remote parts of the body. In the rat and guinea-pig this systemic effect is mediated by somatostatin released from capsaicin-sensitive nerve endings. More recently, a systemic anti-nociceptive effect due to somatostatin release from sensory nerve terminals has also been described (Helyes et al., 2000).

Taken together, capsaicin-sensitive nerve terminals appear to play part in neural regulation of a large scale of organs and tissues in different species. Considering this fact it is surprising that our knowledge concerning the role of capsaicin-sensitive sensory endings in the function of heart musculature and coronary vessels is quite limited (Maggi 1995, Lundberg 1996).

In isolated perfused whole guinea-pig heart, capsaicin provokes the release of tachykinins and CGRP from nerve terminals exerting CGRP-mediated positive inotropic and chronotropic effect (Franco-Cereceda 1988). Tachykinin-CGRP immunoreactive sensory fibres sensitive to capsaicin has also been demonstrated in cardiac tissue obtained from the rats, pigs and humans (Franco-Cereceda 1991a, Maggi 1995). In the atria these fibres are associated with myocardial cells and coronary blood vessels as well as epicardia and endocardia. In the ventricles CGRP positive fibres are close to blood vessels as well as epicardia and endocardia but rarely close to myocardial cells (Franco-Cereceda 1988). Moreover, the functional integrity

of capsaicin sensitive nerve fibres was found to be the prerequisite of inducing ischaemic-preconditioning, the most potent cardioprotective mechanism known to date. In these experiments, the preconditioning phenomenon was lost in isolated hearts obtained from rats pre-treated by systemic doses of capsaicin (Ferdinandy et al., 1997).

Regulation of coronary blood flow

The heart is an aerobic organ, that is most convincingly demonstrated by the fact that its oxygen consumption is 9.7 ml/100g cardiac muscle /min, which is the highest value compared to other organs (Ganong 1993). Under resting conditions, cardiac muscle normally utilises mainly fatty acids for its energy demand instead of carbohydrates. Approximately, 70 % of the normal energy metabolism is being derived from fatty acids (Guyton and Hall 1996). Since fatty acid oxidation is an oxygen dependent process, the appropriate oxygen supply is of crucial importance. Accordingly, blood flow through the coronary arteries is precisely regulated and any imbalance between oxygen supply and oxygen demand can lead to severe clinical conditions such as angina pectoris or heart failure (Daut et al., 1990).

Even in normal resting state, the arteriovenous oxygen difference is as high as 14 ml/100ml blood, meaning that about 70 % of oxygen is extracted as blood passing through the coronary vessels (Ganong 1993). Since the oxygen removal is near maximal at rest, the only way to raise the oxygen supply is to increase blood flow via coronary artery dilation. In fact, a decrease of the oxygen tension (hypoxia) or interruption of blood flow (ischaemia) elicits a marked reduction of resistance of coronary vessels (Daut et al., 1990).

Under physiological circumstances, coronary flow is regulated by both local metabolism of the myocardium and the autonomic nervous system. Oxygen demand is the major factor in local blood flow control. Blood flow increases proportionally to the demand of the cardiac musculature for oxygen (Guyton and Hall 1996). The means by which increased oxygen consumption evokes coronary dilation are not completely understood. It is emphasised by many investigators that a reduction in oxygen concentration liberates vasodilator substances, such as adenosine, adenosine phosphate

compounds, potassium ions, hydrogen ions, carbon dioxide, lactate, bradykinin and prostaglandins (Ganong 1993, Guyton and Hall 1996). Of the compounds released adenosine has the most potent vasodilator property. Asphyxia, hypoxia and intracoronary injections of cyanide all raise coronary blood flow by 200 - 300 % in denervated as well as intact hearts. The characteristic common to these 3 stimuli is hypoxia of myocardial fibres. One final common pathway underlying coronary dilation in response to adenosine and hypoxic stimuli seems to be opening of ATP sensitive potassium channels (K_{ATP}). Hypoxic vasodilatation in isolated, perfused guinea-pig hearts can be prevented, by glibenclamide a K_{ATP} blocker and can be mimicked by a cromakalim, which opens the ATP sensitive potassium channels (Daut et al., 1990).

As it has been mentioned above, the main determinant of coronary blood flow is the oxygen demand of the heart, the nervous control is of secondary importance. The innervation of the heart will be discussed in greater depth later, here those aspect will be mentioned which are indispensable to the understanding of the nervous control of coronary blood flow. Excitation of the autonomic nerves supplying the heart can influence the coronary flow both directly and indirectly (Ganong 1993). The direct effects result from the action of the neurotransmitters released, acethylcholine from the vagal nerves and norepinephrine from sympathetic nerves, on the coronary arteries themselves. Acethylcholine elicits endothelium-dependent relaxation through activation of M1 or M3 receptors located on the endothelial surface resulting in nitric oxide (NO) release (Feletou and Vanhoutte., 1999). Norepinephrine causes vasoconstriction via acting on α_1 receptors leading to a rise in intracellular Ca^{2+} concentration. (Hoffman et al., 1996, Abernethy and Schwartz 1999) The indirect effects results from secondary changes in coronary blood flow provoked by increased or decreased performance of the myocardium.

The indirect effects, which are mostly opposite to the direct ones, have far more important influence on normal control of coronary blood flow. Accordingly, sympathetic stimulation liberating norepinephrine increases heart rate (positive chronotropy) and contractility (positive inotropy) leading to enhanced metabolism. In turn, the increased activity yields vasodilator metabolites (listed above) causing

coronary dilation, despite the direct constrictor effect of norepinephrine. When the indirect positive inotropic and chronotropic effects are prevented by a β adrenergic blocking drug, excitation of sympathetic nerve endings or administration of norepinephrine elicits coronary constriction (Ganong 1993). Conversely, vagal stimulation releasing acethylcholine, slows heart rate (negative chronotropy) and has a slight depressive effect on cardiac contractility (negative inotropy). Both of the effects decrease cardiac metabolism and oxygen consumption leading to indirect coronary constriction in spite of the direct coronary dilator effect of acethylcholine (Guyton and Hall 1996, Hoffman et al., 1996).

It must be stressed again that metabolic factors (hypoxia, lactate, adenosine, prostaglandins, potassium) particularly myocardial oxygen consumption are the major regulators of coronary flow. When the direct effects of nervous stimulation render the coronary flow, the metabolic control of coronary flow generally overrides the direct coronary nervous effects within seconds or minutes under physiological conditions.

Innervation of the heart

General pattern of cardiac innervation

The heart is richly innervated by both vagal (parasympathetic) and sympathetic nerves of a dual efferent and afferent fashion (Pick 1970). Postganglionic sympathetic nerve endings liberate norepinephrine as their principal transmitter and additionally they contain neuropeptide Y (NPY) (Lundberg et al., 1982).The postganglionic cardiac parasympathetic nerves are cholinergic (Loewi 1921). Furthermore, some local parasympathetic ganglion cells in the heart contain vasoactive intestinal polipeptide (Della et al., 1983) and somatostatin (Campbell et al., 1982, Franco-Cereceda et al., 1986).

Sensory nerve fibres in the heart are of both vagal and spinal origin. Fine myelinated (A-delta fibres) and unmyelinated (C-fibres) afferents possess endings in the heart which respond to a wide variety of stimuli and function as nociceptors. The receptors of both types of sensory nerve in the heart are probably polymodal meaning

that they respond to chemical as well as mechanical stimuli (Bishop et al., 1983). A subpopulation of cardiac afferents, presumably belonging to the C-fibre group, can be histologically identified due to their multiple peptide content. These fibres have been suggested to contain substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP).

Vagal afferents

Approximately 80 % of all vagal axons are afferent fibres. The receptors with myelinated vagal afferent fibres are mainly located in the caval vein-atrial junction (Nonidez 1937, Bishop et al., 1983) and seem to be at least partly (Linden 1975) involved in the Bainbridge reflex. Raised atrial pressure evokes an increase in heart rate which can be as high as 75 %. A part of this increase is caused by direct effect of increased volume on sinus node but the vast majority of the increase is elicited by the Bainbridge reflex (Guyton and Hall 1996). The activated stretch receptors of the atria (polymodal receptors) transmit their signals via the vagus nerve to the medulla of the brain. Then, efferent signals are transmitted back through both the vagal and the sympathetic nerves to increase heart rate. This reflex helps to empty the atria. Atrial myelinated afferents (as well as unmyelinated vagal afferents) can, additionally, induce diuretic responses (Goetz et al 1975, Linden 1976, Thoren 1979). The diuretic response consists of two components: afferent arteriolar dilation of the glomeruli and inhibition of antidiuretic hormone secretion from the posterior lobe of the hypophysis (Guyton and Hall 1996) response also known as Henry-Gauer reflex (Schrier and Abraham 1999). Both the afferent arteriolar dilation and decreased antidiuretic hormone secretion result in enhanced urine formation with a resultant decrease in effective plasma volume aiding to maintain the normal blood pressure. Increased arterial wall tension also releases atrial natriuretic peptide inducing copious natriuresis (Ellis et al., 1998). Myelinated vagal afferents also innervate the ventricles (Paintal 1955). Albeit they represent a very small fraction of ventricular receptors (Bishop et al., 1983). These receptors can be activated by increases in myocardial tension and to a certain extent, distension. A subpopulation of these receptors is located along the

coronary arteries (Brown 1965) and respond to changes in coronary perfusion pressure.

A part of vagal cardiac afferents is unmyelinated. Atrial receptors of vagal C-fibres (Coleridge and Coleridge 1977) are located throughout the atria and are usually silent or show a spontaneous discharge at a low frequencies (Thoren 1976). These receptors can also have a cardiac modulated discharge rhythm and the discharge activity is influenced more by atrial distension than contraction (Thoren 1976). Activation of atrial C-fibres appears to elicit vasodilation in the skeletal muscle, skin kidneys and intestines (Bishop et al., 1983). The ventricular unmyelinated vagal afferents (Coleridge et al., 1964, 1979) innervate mainly the left ventricle and very few receptors have been found in the right ventricle (Thoren 1979). Increased volume of the heart and, to a lesser extent increased ventricular contractility influence these receptors. The ventricular vagal C-fibres (Thoren 1979) are involved in eliciting the Bezold-Jarisch reflex (bradycardia and hypotension). Bezold-Jarisch reflex can be provoked experimentally by distension of the left ventricle or injection of serotonin, veratridin or capsaicin into the coronary arteries supplying the left ventricle. Its physiological role is not completely understood but activation of the reflex can account for the hypotension accompanying certain cases of inferior wall myocardial infarction (Ganong .1993). Activation of unmyelinated vagal nerve afferents of both atrial and ventricular origin are also believed to play an important role in the pathophysiology of vasovagal (neurocardiogenic) syncope (Calkins 1999).

Sympathetic afferents

Sympathetic afferents have their cell bodies in the spinal ganglia. Atrial sympathetic myelinated fibres (Malliani et al., 1973, Uchida and Murao 1974b) usually discharge as a single impulse during the cardiac cycle (Recordati et al., 1975, 1976) and thus display a major difference compared to vagal atrial fibres which fire in bursts of impulses. Atrial myelinated fibres of sympathetic origin are activated by contraction as well as stretching (Nishi et al., 1977). Myelinated ventricular sympathetic afferents respond to mechanical stimuli during the cardiac cycle and

enhanced discharge activity is observed when the ventricular pressure is elevated (Malliani 1982).

Atrial unmyelinated fibres (Uchida and Murao 1974b, Malliani 1982) generally show little or no spontaneous discharge activity but are activated by mechanical stimuli (Uchida and Murao 1974a). In contrast to ventricular myelinated fibres which discharge in response to ventricular contractions (Ueda et al., 1969, Malliani et al., 1973), there is often no correlation between impulse activity in unmyelinated ventricular sympathetic fibres and ventricular dynamics (Uchida and Murao 1974a, 1975). Unmyelinated fibres are excited by ventricular distension and stimuli evoking positive inotropic effects (Casati et al., 1979)

As far as the physiological role of sympathetic cardiac afferents are concerned scant data are available (Malliani 1982). Sympathetic pathways have been suggested to be involved in the Bainbridge reflex (Gupta 1975, Bishop et al., 1976) and to possess excitatory effect on renal sympathetic nerve activity and arterial blood pressure (Weaver 1977, Malliani 1982). More importantly, sympathetic cardiac afferents are believed to account for sensation of cardiac pain (Lindgren and Olivecrona 1947, White 1957).

Sympathetic C-fibres can be activated by a variety of chemical substances such as bradykinin, potassium, (Uchida and Murao 1974b) digitalis glycosides and lactic acid (Coleridge and Coleridge 1979, Bishop et al., 1983). Both myelinated (Brown 1967, Malliani et al., 1973, Uchida and Murao 1974a) and unmyelinated (Uchida and Urao 1974a, Casati et al., 1979) ventricular sympathetic fibres are stimulated by impairment of the coronary blood flow. Coronary arterial occlusion and myocardial ischaemia thus excite sympathetic as well as vagal afferents (Bishop 1983). Cardiac ischaemia may be accompanied by vagally mediated inhibitory reflexes or sympathetically mediated excitatory reflexes. The nature of the reflex response elicited by coronary obstruction seems to be dependent on the localisation of the ischaemia. Accordingly, anterior infarction may lead to excessive sympathetic activity with tachycardia and/or hypertension whereas inferior infarctions often can evoke vagal overreactivity resulting in bradycardia and or hypotension (Pantridge 1978).

Local efferent role of capsaicin-sensitive sensory neurones in the heart

Having overviewed the role of the afferent part of the vagal and sympathetic innervation, over the rest of the paper I shall focus on the local efferent function of the polymodal C-fibres with respect to the heart. As I have mentioned in the general introduction, capsaicin-sensitive nerve terminals play part in eliciting local efferent responses in a wide variety of organs. In term of the heart, our knowledge available about the role of capsaicin-sensitive neurones is far from complete and relies mainly on experiments performed by the group of Franco Cereceda.

In the isolated atrium from guinea pig heart, capsaicin induces positive inotropic and chronotropic effects (Fukuda and Fujiwara 1969, Molnar et al., 1969, Lundberg et al., 1984). These responses were absent in animals which have been systematically pre-treated with capsaicin (Lundberg et al., 1984) which causes degeneration of sensory nerves in the heart (Papka et al., 1984). This suggested that capsaicin released mediator(s) from sensory nerve endings damaged by systemic capsaicin pre-treatment. Subsequently, capsaicin-induced effects were mimicked by CGRP (Franco-Cereceda and Lundberg et al., 1985) and blocked by $CGRP_{(8-37)}$ an antagonist of CGRP (Franco-Cereceda 1991b). Tachykinin-CGRP immunoreactive sensory fibres sensitive to capsaicin have also been demonstrated in cardiac tissues obtained from the rats, pigs and humans. (Franco-Cereceda 1991ab, Maggi 1995). The myocardium of the atria had the highest density of CGRP containing fibres while in the ventricles CGRP positive fibres were mainly associated with coronary vessels.

The functional integrity of capsaicin sensitive nerve fibres was found to be the prerequisite of inducing ischaemic-preconditioning, the most potent cardioprotective mechanism known to date. In these experiments, the preconditioning phenomenon was lost in isolated hearts obtained from rats pre-treated by systemic doses of capsaicin (Ferdinandy et al., 1997).

Considering the high prevalence and incidence of cardiac diseases (myocardial infarction, angina pectoris, arrhythmias) and the above experimental data with particular respect to the role of sensory neurones, I felt challenging to further

investigate the function of capsaicin-sensitive nerve endings in isolated rat and guinea-pig hearts under physiologic and clinically relevant pathologic conditions.

Our own findings

Experiments performed with isolated guinea-pig hearts

We started the experiments with investigating to what extent capsaicin-induced effects were dependent on the NO synthesis in isolated Langendorff-perfused guinea-pig hearts. As I have described above, the CGRP mediated positive inotropic and chronotropic effects of capsaicin have been known for a long time (see above). More recently, evidence has been provided for the co-localisation of CGRP and NO in sensory nerves of the heart (Sosunov et al., 1995). An article appeared in 1997 reporting the possible involvement of NO in the vasodilator effect of capsaicin (Mitchell et al., 1997) in rabbit coronary circulation. They found that the nitric oxide synthase inhibitor NG nitro L-arginin methyl ester blocked the effect of capsaicin but not that of CGRP. They concluded that CGRP release following sensory nerve activation was dependent on the functional integrity of NO synthesis (Mitchell et al., 1997). By contrast, another work published about at the same time evidenced that the effect but not the liberation of CGRP was dependent on intact NO synthesis in human internal mammary arteries (Raddino et al., 1997).

We used isolated Langendorff-perfused guinea pig hearts to test the NO dependency of changes in heart rate and coronary flow in response to capsaicin (Oroszi et al., 1999a). Our findings were in accordance with previous observations (Franco-Cereceda 1991a,b) showing that capsaicin enhanced coronary flow and increased heart rate (Oroszi et al., 1999a). Furthermore, our result clearly demonstrated the NO dependent manner of capsaicin effects. In the presence of N_{ω}-nitro-L arginine methyl ester (L-NAME), a non-selective NO synthase inhibitor (Keskil et al., 1999) capsaicin-elicited cardiac and coronary responses were lost. When L -Arg was administered later (the concentration of which was 100 times greater than

that of L-NAME) capsaicin-evoked effects reappeared (Oroszi et al., 1999a). The recurrence of capsaicin-elicited response after L-Arg addition was prevented by $hCGRP_{(8-37)}$,a CGRP receptor antagonist.

Based on these results we not only reinforced earlier observations, but we provided evidence for the NO dependency of capsaicin-induced positive inotropy and chronotropy in guinea pig heart. Unfortunately, whether the NO is required for the release of CGRP or it contributes to the effect of CGRP can not be judged according to this series of experiment due to the nonselective nature of the NO synthase inhibitor applied. Further experiments, using more specific (neural) inhibitors will help to answer the question.

Intact capsaicin-sensitive sensory innervation is obligatory to elicit pacing-induced preconditioning in isolated rat heart (Ferdinandy et al., 1997) raising the possibility that CGRP and NO of neural origin may underlie at least in part the mechanism of pacing-induced preconditioning. Development of vascular nitrate tolerance has been shown to lead to loss of preconditioning in rabbits (Szilvassy et al., 1994). Coronary vasodilatation along with an increase in heart rate have been demonstrated to be dependent on the production of NO in isolated Langendorff-perfused guinea-pig heart (Oroszi et al., 1999a). Based on these results, we hypothesised that in state of vascular nitrate tolerance in isolated guinea-pig hearts capsaicin-induced changes were deteriorated. Vascular nitrate tolerance is of great clinical importance due to the high prevalence of chronic stable angina pectoris and congestive failure, two conditions in which nitrate therapy has been approved and recommended (Parker 1998). Various theories have been put forward to account for the development of nitrate tolerance but none of them has proved to be capable of explaining it completely (Parker 1998). To test our hypothesis we provoked vascular nitrate tolerance in guinea-pigs (Oroszi et al., 1999b). Under experimental conditions, development of haemodynamic nitrate tolerance can be confirmed by the lack of decrease in mean arterial blood pressure in response to intravenous bolus injection of 30 μg/kg nitro-glycerine (Szilvassy et al., 1994, Oroszi et al., 1999b). In state of nitrate tolerance capsaicin failed to elicit an increase either in heart rate or coronary flow verifying our hypothesis. It must be emphasised again, that the level at which nitrate

tolerance interferes with the effect of capasicin can not be determined. Nitrate tolerance can affect both the release and the effect of CGRP. However, viewing the importance of haemodynamic nitrate tolerance clinically and the suggested role of CGRP in human epicardial coronary arteries (Franco-Cereceda 1991a) further experiments elucidating the exact mechanism of the process seems to be promising and clinically relevant.

In summary, our investigations on guinea-pig hearts have yielded results which were in accordance with previous data, furthermore, beyond confirming earlier observation, we could demonstrate for the first time that capsaicin-elicited effects in this species were NO dependent and deteriorated in state of haemodynamic nitrate tolerance.

Experiments with isolated rat hearts

The vast majority of investigations dealing with function of capsaicin sensitive sensory nerve endings has been carried out on isolated guinea-pig preparations (see above). The direct pharmacological effects of capsaicin with respect to rat heart have not been extensively examined (Szolcsanyi 1996). Accordingly, after completing the experiments with guinea-pig hearts we focused our attention on clarifying the possible role of capsaicin-sensitive sensory nerve terminals in isolated rat heart.

Initially, we expected similar responses to capsaicin in both species. By contrast, during the course of the first series of experiments, the Langendorff-perfused rat hearts displayed not positive chronotropy and reduced coronary perfusion pressure - as would have been predicted based on previous data (Franco-Cereceda 1988) - but a marked decrease of coronary flow and a drop in heart rate in response to capsaicin. These results were consistent in further experiments, therefore, the mechanism and particularly the possible role of neural elements in this new type of capsaicin response were analysed in detail.

Subsequently, we measured the cardiac parameters in response to capsaicin in isolated working rat hearts, in which not only coronary flow and heart rate but aortic

flow, left ventricular developed pressure (LVDP) and it first derivative ($LVdP/dt_{max}$) could be recorded (Tosaki and Hellegouarch 1994). All cardiac parameters were deteriorated in hearts challenged by capsaicin in a dose-dependent manner (Szolcsanyi et al., 1999, Table 1).

On the basis of the observations listed above, we hypothesised that the pronounced decrease in coronary flow was the primary change leading to the decline of all the cardiac functions studied. Reduction of the coronary flow in response to 1μM capsaicin was as high as 75 % and practically irreversible (Szolcsanyi et al., 1999, Table 1). Since all experiments have been performed with isolated rat hearts, involvement of any systematically produced potent vasoconstrictor substance could be ruled out. Taking into account the selective site of action of capsaicin on sensory nerve endings (Szolcsanyi 1996) along with recent evidence showing its cloned receptor, the so called vanilloid receptors not to be present on cardiac myocytes or endothelial cells (Caterina et al, 1997) we presumed that the mediator release was of neural origin. The next step was to find a putative mediator present in capsaicin-sensitive sensory neurones possessing potent long-lasting vasoconstrictive property that might be liberated in response to capsaicin.

With regard to the heart no data were available of a compound fulfilling these requirements. We noticed that the coronary constrictor effects of capsaicin were strikingly similar to those seen with endothelin in dog coronary vessels (Juhasz-Nagy 1996) both in term of potency and duration. Endothelin, however, has not been considered as a neuropeptide and no evidence suggested its localisation in capsaicin-sensitive afferents (Holzer 1991, Szallasi and Blumberg 1999). Some data indicated its presence in nerve fibres. Endothelin 1 mRNA and endothelin-like immunoreactivity were shown in human dorsal root ganglia a long time ago (Giaid et al., 1989) but these observations have not been supported by any functional evidence. Endothelin-like immunoreactivity has been reported in dorsal root ganglia of the guinea-pig (Franco-Cereceda et al., 1991) and in the sensory ganglia of the pig (Hemsen and Lundberg 1991). Recently, perivascular nerves immunolabelled with polyclonal antibody to endothelin 1 have also been demonstrated in rat basillary artery (Loesch et al., 1998), again without any functional approach.

Taking these publications into account, endothelin seemed to be the candidate regarding its potent vasoconstrictive nature. Therefore, we co-administered capsaicin with PD142893, a nonselective endothelin receptors antagonist to test our presumption. The results strongly suggested the role of endothelin as the mediator concerned, since PD142893 near totally abolished the capsaicin-induced increase in coronary resistance and prevented the deterioration of all the cardiac parameters monitored (Szolcsanyi et al., 1999) In a separate setting, hearts were perfused with 0.1 nM endothelin in the absence or presence of PD142893. Of interest, the percentage decrease of all cardiac functions in response to exogenous endothelin were near indentical with those seen with capsaicin (Szolcsanyi et al., 1999, Fig 1) both in the absence and presence of the nonselective endothelin receptor blocking agent. The fact that exogenous endothelin mimicked the cardiac responses of capsaicin further substantiated our mediator hypothesis.

In the light of the above evidence, we concluded that the cardiac effects of capsaicin were likely to be mediated by endothelin release in isolated working rat heart. It was clear, however, that the evidence was circumstantial based on functional observations. We did not have direct biochemical data documenting the liberation of endothelin and the source of endothelin was suspected of neural origin (see above) but participation of the endothelial source cold not be excluded unequivocally. These shortcomings prompted us to continue our work.

At first, we tried to provide direct biochemical evidence for the release of endothelin. In Langendorff-perfused rat hearts we could demonstrate that capsaicin-elicited a concentration-dependent liberation of endothelin in the coronary effluent (Szolcsanyi et al., 2000, Fig 2) These measurements were performed in co-operation with the Molecular Biology Department in Berlin.

Whether or not endothelin had been freed from sensory neurones was of crucial importance in view of the limited data available in this respect (see above). Relying on data found in the literature Ca^{2+} dependency of neuropeptide release seemed to be exploitable tool. In guinea-pig Langendorff-perfused hearts capsaicin released CGRP in calcium-dependent fashion (Franco-Cereceda 1988). Similarly, in the human LAD capsaicin provoked CGRP release was reported to be calcium-dependent (Franco-

Cereceda 1991a). Accordingly, we perfused rat hearts in presence of normal (2.4 M) and reduced (1.2 and 0.6 M) extracellular calcium concentration $[Ca^{2+}]_o$. Decreased $[Ca^{2+}]_o$ per se was without effect on the baseline parameters. By contrast, in presence of 0.6 M $[Ca^{2+}]_o$ capsaicin-elicited effects were completely abolished indicating the Ca^{2+} dependency of endothelin release (Szolcsanyi et al 2000 Table1). On the other hand, responses to endothelin were not affected by the drop in $[Ca^{2+}]_o$. In this series of experiments we perfused hearts with 0.1 nM endothelin in presence of low $[Ca^{2+}]_o$ (0.6 M). The effects of endothelin seen with normal and low $[Ca^{2+}]_o$ did not differ significantly. Taken together, the blunted effect of capsaicin in presence of low $[Ca^{2+}]_o$ was assumed to be due to diminished endothelin release from sensory neurones.

Another possible source of endothelin were the endothelial cells, lining the coronary blood vessels, though the involvement of which seemed unlikely (see above). In order to obtain clear-cut evidence about the role of endothelium we destroyed it chemically. We used Triton X-100, a detergent widely applied to destroy the functional endothelium (Kamata et al., 1996). After destroying the functional endothelium, the capsaicin-elicited decrease in coronary flow remained unchanged (Szolcsanyi et al., 2000) evidencing that not the endothelium was the source of the endothelin.

Considering, that mainly endothelin A receptor was believed to account for vasoconstriction (Stjernquist 1998) both capsaicin and endothelin were co-perfused with BQ-123, a selective endothelin A receptor antagonist. BQ-123 completely antagonised the effects of both capsaicin and endothelin showing that the cardiac effects of capsaicin were mediated through endothelin A receptor.

Based on the results detailed above, we concluded, that the capsaicin-elicited cardiac effects in the isolated rat heart were mediated by endothelin of neural origin acting on endothelin A receptors.

The remarkable difference in capsaicin-elicited responses between isolated perfused heart preparations obtained from guinea pig and rat is unequivocal and seems to be underlain by release of two mediators of opposing effect, CGRP and endothelin, respectively. However, the reason why predominantly endothelin is freed from sensory nerurones in rat and CGRP in guinea-pig heart is elusive. We speculate that

differences in the species can account for the disparity observed. This assumption seems to be supported by the fact that endothelin-like immunoreactivity has also been demonstrated in dorsal root ganglia of the guinea-pig (Franco-Cereceda et al., 1991). More importantly, in release experiments with cultured dorsal root ganglia cells obtained from guinea-pigs, potassium, capsaicin or antidromic nerve stimulation failed to increase the concentration of endothelin in the effluent whereas CGRP level was raised severalfold (Franco-Cereceda et al., 1991). Considering that CGRP like immunoreactivity has been shown to be present in rat neuronal tissues including the heart (see above) we assume that the proportion of the mediators released might be different. In the guinea-pig heart CGRP is freed to greater extent dominating the cardiac responses observed. By contrast, in rat heart endothelin is released to a higher degree determining principally the responses evoked by capsaicin masking the possible opposing effects of CGRP.

Diabetic rat hearts

Decreased sensory neuropeptide release such as CGRP, substance P and somatostatin evoked by electrical field stimulation has been shown to be characteristic of diabetic neuropathy (Nemeth et al., 1999a). Recently, it has been suggested to account for deterioration of several adaptive mechanism such as neurogenic inflammation (Walmsley and Wiles 1991; Gyorfi et al., 1996, Nemeth et al., 1999b). and the ability of the heart to adapt to repetitive ischaemic challenges i.e. the ischaemic preconditioning phenomenon (Ferdinandy et al., 1997). Capsaicin acting on vanilloid receptors located on a subpopulation of primary afferent neurones has been demonstrated by our group to decrease coronary flow with resultant deterioration of cardiac functions underlain by neural endothelin release in isolated working rat hearts (Szolcsanyi et al., 1999, 2000).

Based on these results we studied whether 4 and 8-week diabetes mellitus evidenced by combined neuropathy evoked by streptozotocin influenced the effects of capsaicin on isolated Langendorff-perfused rat hearts.

Our results demonstrated unequivocally that the effects attained by capsaicin (a decrease in both coronary flow and heart rate) were preserved in 4 but lost in 8-week diabetic rat hearts (Nemeth et al., 2000). Since, according to our results the capsaicin-released endothelin was of neural origin, impairment of cardiac sensory neurones due to the long-lasting diabetic state could account for the lost cardiac effects.

Summary

The present work was devoted to elucidating the role of capsaicin-sensitive nerves of the isolated heart in guinea-pig and rat under physiologic conditions and in experimental diabetes or nitrate tolerance.

In the guinea-pig heart, we corroborated all the results presented previously, in addition, we could provide evidence for the NO dependency of capsaicin-induced positive inotropy and chronotropy. Furthermore, in state of nitrate tolerance, a condition characterised by deficient endogenous NO production, capsaicin-elicited coronary dilation and increase in heart rate has also been found to be deteriorated.

The main novelty of our work is the description and clarification of the responses evoked by capsaicin challenge in rat hearts which shed light on the sensory neuropeptide role of endothelin. In this respect we demonstrated that:

1. In isolated working rat heart and Langendorff preparations, capsaicin produces a concentration-dependent increase in coronary resistance with resultant deterioration of the cardiac parameters monitored.
2. Exogenous endothelin mimics the effect of capsaicin in isolated working rat heart.
3. The cardiac effects of both capsaicin and exogenous endothelin can be prevented by PD 142893, a non-selective endothelin receptor antagonist.
4. BQ-123, a selective endothelin A receptor antagonist at a concentration of 10^{-6} mol/l inhibited the effects of both capsaicin and exogenous endothelin.
5. Capsaicin elevates the level of endothelin in coronary effluents collected in Langendorff-perfused rat heart.
6. Capsaicin-induced increase in coronary resistance is not dependent on the functional integrity of the endothelium.

Taken together, cardiac effects of capsaicin on isolated rat heart seem to be mediated by the release of endothelin. We conclude that endothelin is released not from the endothelium but from the sensory nerve endings on which capsaicin receptors are located.

Under 8-week diabetic conditions, we could document the impairment of cardiac sensory nerves by the lack of neural endothelin liberation in response to capsaicin.

With respect to the possible clinical implication of our results, in particular about the possible role of neural endothelin in cardiovascular diseases it is too early to predict anything. Nevertheless, it is worth mentioning, that recently an increased endothelin expression has been observed in perivascular nerves of spontaneously hypertensive rats (Milner et al., 2000).

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INTERPLAY BETWEEN NITRIC OXIDE AND CGRP BY CAPSAICIN IN ISOLATED GUINEA-PIG HEART

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Capsaicin at a concentration of 10^{-7} M induced a significant increase in heart rate and increased coronary flow in isolated Langendorff-perfused guinea-pig hearts. This effect was completely blocked by 30 μM of N_ω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase. Additional incubation with 3 mM L-Arg antagonized the inhibitory effect of L-NAME. In the presence of 1 μM of a human calcitonin gene-related peptide fragment (hCGRP 8-37), a CGRP-receptor antagonist, L-Arg was without effect. We conclude that a capsaicin-induced increase in coronary flow and heart rate is dependent from an interplay between CGRP and NO in guinea-pig hearts.

KEY WORDS: capsaicin, calcitonin gene-related peptide, nitric oxide, guinea-pig heart.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) underlies an increase in heart rate and vasorelaxation produced by capsaicin in isolated guinea-pig hearts and porcine coronary arteries, respectively [1–3]. The coronary vasorelaxation response originally was described to be an endothelium-independent one [2], however, inhibition of the nitric oxide (NO) cyclic GMP pathway, by either blockade of NO synthesis [4] or development of nitrate tolerance [5] has been shown to block cardioprotection conferred by preconditioning, an adaptive mechanism dependent from the integrity of capsaicin-sensitive cardiac nerve function [6]. Recently, Mitchell *et al.* [7] found that capsaicin-induced reduction of coronary perfusion pressure was sensitive to blockade of NO synthesis in rabbits, a finding confirmed by Raddino *et al.* [8]. Moreover, evidence has been provided for a colocalisation of CGRP and NO synthase in sensory nerves of the heart [9]. Therefore, the present work was concerned with the possibility that NO was involved in CGRP-mediated coronary vasodilation by capsaicin. To test this hypothesis, we used isolated Langendorff-perfused guinea-pig hearts in which the effects of capsaicin are well characterized.

METHODS

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical committe of the Medical University of Pecs, Hungary.

Methods have been described in detail elsewhere [10]. In brief, hearts from adult male guinea-pigs (350–400 g) were mounted on a Langendorff apparatus. Following a 10-min period of aerobic perfusion, the hearts were exposed to 10^{-7} M capsaicin for 5 min. The perfusion medium consisted of a modified Krebs–Henseleit bicarbonate buffer (in mM: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulfate 1.2 and glucose 10). In the next series of experiments, 30 mg kg^{-1} of N_ω-nitro-L-arginine methyl ester (L-NAME) a non-selective NO synthase inhibitor was injected

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i.p. into the animals 30 min prior to heart excision. The hearts were then perfused with 30 μM L-NAME for 20 min which was succeeded by perfusion with 10^{-7} M capsaicin for 5 min. This was followed by additional perfusion with 3 mM L-Arg. In the third protocol, 1 μM of hCGRP 8-37, a CGRP receptor antagonist was administered 10 min preceding the perfusion with L-Arg. In a separate set of experiments, 1 μM of hCGRP 8-37 was perfused with and without capsaicin. During all the protocols changes in heart rate and coronary flow were measured.

Drugs and chemicals

All drugs used in this study were purchased from Sigma (St Louis, MO, USA). Constituents of the modified Krebs–Henseleit solution were bought from Merck (Darmstadt, Germany). Capsaicin stock solution was made as follows: capsaicin was dissolved in 10% ethanol, 10% Tween 80, and 80% saline (0.9% w/v NaCl). This stock solution was diluted to the concentrations applied with modified Krebs–Henseleit solution. All the other drugs were dissolved in modified Krebs–Henseleit solution.

Statistical analysis

Data expressed as means ± SE of the means (SEM) were evaluated by means of analysis of variance (ANOVA) followed by a modified Student's t-test for multiple comparisons according to Bonferroni's method. Changes were referred to as significant at $P < 0.05$.

Exclusions

Hearts exhibiting ventricular fibrillation or severe ventricular arrhythmias during the period of aerobic perfusion were excluded from further examinations.

RESULTS

Capsaicin induced a significant increase in coronary flow, an effect blocked by the combined pretreatment with L-NAME. This inhibitory effect of L-NAME was lost subsequent to a 30-min additional perfusion with L-Arg. Nevertheless, L-Arg did not reverse the inhibitory effect of L-NAME in the presence of the hCGRP 8-37 fragment. The CGRP receptor antagonist attenuated capsaicin-induced increase in coronary flow, but it was without effect on baseline values (Fig. 1). The solvent of capsaicin was without effect.

Capsaicin-induced increase in heart rate was significantly attenuated in hearts from animals pretreated with L-NAME, an effect reversed by concurrent perfusion with L-Arg. In the presence of the hCGRP 8-37 fragment, however, L-Arg was without effect. Capsaicin did not increase heart rate in the presence of hCGRP 8-37 (Fig. 2). The heart rate was not modified by the solvent of capsaicin.

DISCUSSION

The results indicate that capsaicin induces an in-

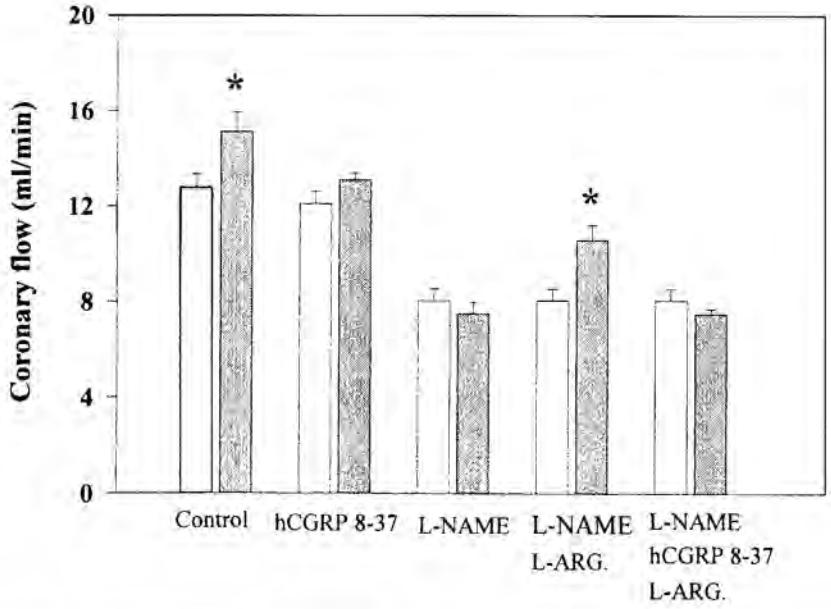

Fig. 1. Effect of capsaicin at a concentration of 10^{-7} M on coronary flow in isolated Langendorff-perfused guinea-pig hearts. Open columns represent values without capsaicin, hatched columns indicate values obtained with capsaicin, in the presence of normal Krebs (first pair of columns), 1 μM hCGRP 8-37 (second pair of columns), 30 μM L-NAME (third pair of columns), 30 μM L-NAME + 3 mM L-Arg (fourth pair of columns) or 30 μM L-NAME + 1 μM hCGRP 8-37 + 3 mM L-Arg (fifth pair of columns). The data are expressed as means ± SEM ($n = 5$). *Significant difference between values obtained 'with *vs* without capsaicin' at $P < 0.05$.

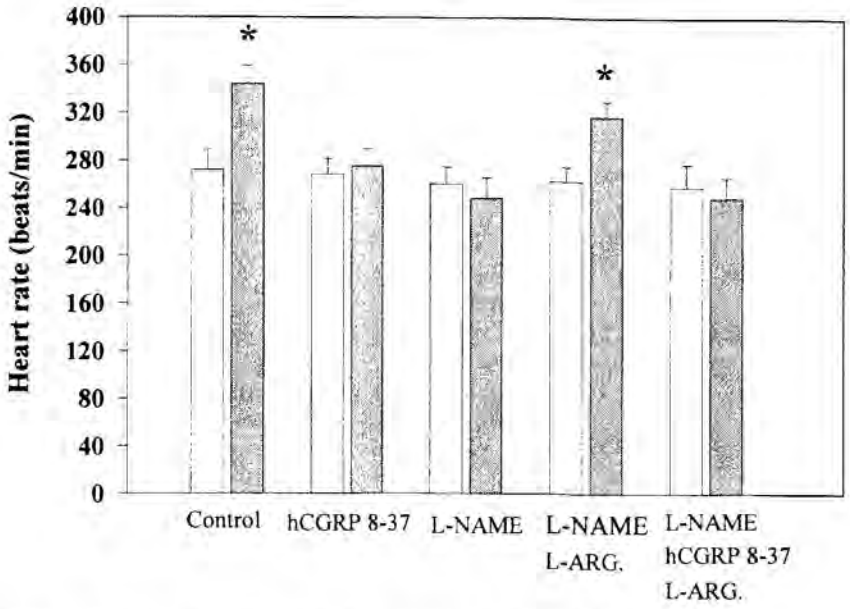

Fig. 2. Changes in heart rate elicited by capsaicin at a concentration of 10^{-7} M in the presence of normal Krebs solution (first pair of columns), 1 μM hCGRP 8-37 (second pair of columns), 30 μM L-NAME (third pair of columns), 30 μM L-NAME + 3 mM L-Arg (fourth pair of columns), 30 μM L-NAME + 1 μM hCGRP 8-37 + 3 mM L-Arg (fifth pair of columns). Open columns: baseline values, hatched columns: values obtained after capsaicin. The data are expressed as means ± SEM ($n = 5$). *Significant difference between values obtained 'with *vs* without capsaicin' at $P < 0.05$.

crease in both heart rate and coronary flow in isolated Langendorff-perfused guinea-pig hearts. These effects are dependent from endogenous NO production, since pretreatment with L-NAME, a non-selective inhibitor of nitric oxide synthase abolished these effects of capsaicin. Nevertheless, CGRP receptor blockade was also found to block the reversal of the effects of L-NAME by L-Arg excess, suggesting a cooperative effect of CGRP and NO in positive chronotropy and an increase in coronary flow by capsaicin at least in the guinea-pig heart.

These results are consistent with previous observations that capsain enhances coronary flow and exerts a positive chronotropic effect in isolated guinea-pig heart [2]. These effects were suggested to be mediated by CGRP, since they were either mimicked by CGRP or antagonized by hCGRP 8-37, a CGRP receptor blocking agent [2]. The CGRP-mediated vascular effects of capsaicin have been interpreted as those developing independent of the endothelium derived relaxing factor (EDRF), as methylene blue, an inhibitor of soluble guanylate cyclase in vascular smooth muscle cells was without effect on the capsaicin response in porcine coronary arteries. However, EDRF does not exclusively involve substance(s) that act through guanylate cyclase, since relaxing factors liberated by the endothelium other than NO, such as prostacyclin and/or endothelium derived hyperpolarizing factor (EDHF) act through cyclic AMP and opening of potassium channels, respectively. Moreover, methylene blue is not specific for vascular soluble guanylate cyclase. Therefore, either positive or negative results obtained with methylene blue are not suggestive for possible implication of endothelial function in any cardiovascular effects.

Moreover, some of the vasorelaxant effects of NO are mediated by pathways independent of the cyclic GMP system. In addition, NO synthase is present not only in endothelium but also in sensory nerves and in vascular smooth muscle [9, 11]. Since a bulk of evidence has been provided for colocalisation of NO synthase and various neuropeptides including CGRP in sensory nerve endings, it was not surprising that the integrity of NO synthesis was found to be of dominant influence on CGRP-dependent coronary artery dilation by capsaicin in rabbits [7, 8]. Nevertheless, since the effects of capsaicin on coronary vascular tone were described in hearts from guinea-pigs, a species highly sensitive to both the neurotransmitter-releasing and neurotoxic effects of capsaicin, we have chosen the very simple experimental model of the Langendorff-perfused isolated heart to test NO dependency of the capsaicin effect. According to our best knowledge, this study provides the first evidence for the interaction between endogenous NO and CGRP in guinea-pig heart. Based on the present results, however, it is not possible to verify as to whether NO synthesis is a primary response to capsaicin with an ensuing release of CGRP or the capsaicin-induced CGRP release is accompanied by a concomitant increase in NO synthesis that contributes to the '*per se*' vasorelaxant effect of

the peptide. If the latter were the case, the vasorelaxation response to CGRP would be attenuated by inhibition of NO synthesis, whereas a direct measurement of CGRP from coronary effluent would indicate a possible influence of a NO donor on CGRP release. Accordingly, the study can not verify the source of NO produced in response to capsaicin. Notwithstanding, capsaicin acts on particular receptors, the clone of which is exclusive for sensory nerves [12], thus a neural origin of NO preceding or supporting the effect of CGRP is likely.

Whatever the mechanisms, the results suggest, that the sensory effector function of capsaicin-sensitive nerves may promote significant coronary vasodilatory effects implying an interplay between CGRP and the L-Arg, NO pathway. Since NO-deficient states have been shown to underlie a series of cardiovascular complications of epidemiologically significant diseases or certain conditions such as diabetes mellitus, atherosclerosis, and insulin resistance, or aging, pharmacologic support of the L-Arg, NO pathway may confer protection on patients at risk of ischaemic heart disease even at levels of sensory nerve function.

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Rapid communication

Interaction between capsaicin and nitrate tolerance in isolated guinea-pig heart

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Abstract

Capsaicin-induced increases in heart rate and coronary flow were blocked by N^G-nitro-L-Arg-methyl ester (30 mM) in Langendorff-perfused guinea-pig hearts. Neither heart rate nor coronary flow changed by capsaicin in hearts from animals made tolerant to the hypotensive effect of 30 μg/kg nitroglycerin by the administration of 50 mg/kg nitroglycerin subcutaneously 4 times a day over 3 days. We conclude that the effector function of sensory nerves may deteriorate in nitrate tolerance.

Keywords: Capsaicin; Coronary flow; Nitrate tolerance

Nitric oxide (NO) in concert with calcitonin gene-related peptide (CGRP) have been shown to be involved in capsaicin-induced coronary vasodilation and reduction in coronary perfusion pressure in rabbits and pigs (Mitchell et al., 1997; see for review Franco-Cereceda, 1988). If NO was of dominant influence in neurogenic coronary artery dilation, we postulated that either pharmacologic blockade of NO synthesis or desensitization of the effects of endogenous NO by nitrate tolerance would influence capsaicin-induced coronary dilation. We therefore sought whether development of haemodynamic tolerance to nitroglycerin influenced the decrease in coronary resistance and positive chronotropy evoked by capsaicin (see for review Franco-Cereceda, 1988) in Langendorff-perfused guinea-pig hearts.

These experiments conform with the European Community guiding principles for the care and use of laboratory animals and the experimental protocol applied has been approved by the ethical committee of our university.

Adult male guinea-pigs weighing 350–450 g were randomized into two groups. Six animals were given 50 mg/kg nitroglycerin s.c. (EGIS, Budapest, Hungary) 4 times a day over 3 days to induce haemodynamic nitrate tolerance (Szilvássy et al., 1994). The development of tolerance was confirmed by the lack of decrease in mean arterial blood pressure in response to an intravenous bolus of 30 μg/kg nitroglycerin (Szilvássy et al., 1994) in pentobarbitone (30 mg/kg i.p.)-anaesthetized animals. In the control group, (12 guinea-pigs) 150 ml/kg ethanol was diluted with distilled water (2 ml); the solvent for nitroglycerin was administered in the same way.

Six hearts from normal animals were then excised and mounted on a Langendorff apparatus as described (Tosaki et al., 1993). Following a 20 min period of aerobic perfusion with Krebs solution, the hearts were exposed to 0.1 mM capsaicin for 5 min. Separate hearts (n:6) were perfused with 30 mM N^G-nitro-L-Arg-methyl ester, a NO synthase inhibitor over 30 min preceding the exposure to capsaicin (0.1 μM). This was followed by perfusion with L-Arg (3 mM). The hearts from 'tolerant' animals were exposed to capsaicin after the 20 min equilibration period. Changes in heart rate and coronary flow were measured as described (Tosaki et al., 1993). All drugs and chemicals

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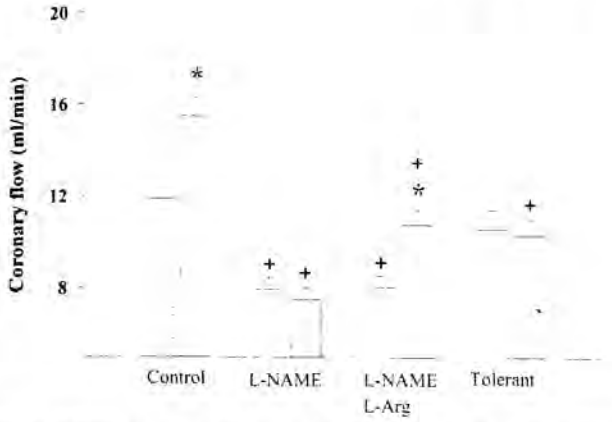

Fig. 1. Effect of capsaicin (0.1 μM) on coronary flow in isolated Langendorff-perfused guinea-pig hearts. Open columns represent values 'without capsaicin', hatched columns indicate values 'with capsaicin'. Control refers to as perfusion with Krebs solution; L-NAME: after perfusion with 30 mM N^G-nitro-L-Arg-methyl ester; L-NAME, L-Arg: additional perfusion with 3 mM L-Arg; Tolerant: the hearts were excised from animals made tolerant to the hypotensive effect of 30 mg/kg nitroglycerin i.v. The data are means ± S.E.M. obtained with 6 preparations. * designates a significant difference between values with vs. without capsaicin at $P < 0.05$, +, significantly different from corresponding control at $P < 0.05$.

were purchased from Sigma (St. Louis, MO) except nitroglycerin which was a gift from EGIS (Budapest, Hungary). The data expressed as means ± S.E.M. were analyzed with one-way analysis of variance (ANOVA) followed by Bonferroni's *t*-test. Level of significance was $P < 0.05$.

The capsaicin-induced increase in heart rate (25 ± 3.9%) and coronary flow were blocked by N^G-nitro-L-Arg-methyl ester in hearts from the normal animals (Fig. 1). These inhibitory effects of were reversed by L-Arg. Interestingly, L-Arg did not restore baseline coronary flow decreased by N^G-nitro-L-Arg-methyl ester. Neither heart rate nor coronary flow changed by capsaicin in hearts from the 'tolerant' animals (Fig. 1).

The results confirm previous findings in that capsaicin increases heart rate and coronary flow in Langendorff-perfused guinea-pig heart (see for review Franco-Cereceda, 1988). These effects are NO-mediated as indicated by the effect of N^G-nitro-L-Arg-methyl ester reversible by L-Arg excess. The major original finding of this work, however, is that the capsaicin effects are lost in haemodynamic nitrate tolerance.

Capsaicin-sensitive sensory nerves influence cardiovascular function due to their neurotransmitters such as CGRP and NO underlying their local effector function (see for review Franco-Cereceda, 1988). These nerve endings act as sensors for ischaemia, hypoxia, lactate, extracellular K^+, responding to signals with neurotransmitter release (see for review Franco-Cereceda, 1988). We have shown that depletion of the CGRP and NO contents of these nerves blocks preconditioning, the most effective cardioprotective mechanism described to date (see for review Ferdinandy et al., 1998). Nitric oxide seems to be the dominant mediator in this respect, since either blockade of NO synthesis or desensitization of the effects of endogenous NO by development of nitrate tolerance also blocked preconditioning (Szilvássy et al., 1994; see for review Ferdinandy et al., 1998). Here we show that the effect of pharmacological activation of capsaicin-sensitive nerves is also vulnerable to both blockade of NO synthesis and nitrate tolerance. Tolerance to nitroglycerin in vivo may modulate vascular effects of endogenous NO due to endothelial superoxide production that scavenges NO yielding peroxynitrate. However, no deficiency in NO production from nitroglycerin has been found in rats with tolerance to nitroglycrin in vivo (Laursen et al., 1996), suggesting the possible implication of mechanisms distal to NO metabolism. Moreover, baseline coronary flow was not altered by nitrate tolerance in our study suggesting effective compensatory mechanisms by endothelium-dependent vasodilators other than NO such as endothelium derived hyperpolarizing factor or prostacyclin. This might explain the virtual contradiction to results by Yaoita et al. (1994) who found that capsaicin-sensitive neuropeptides substantially contributed to baseline coronary flow in rats. Since the effect of capsaicin is selective for sensory neurons (Caterina et al., 1997), it is likely that beyond direct vascular effects, nitrate tolerance may cause profound cardiovascular regulatory disorders implying sensory nerves.

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Short communication

Endothelin release by capsaicin in isolated working rat heart

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Abstract

Capsaicin (1 nM–1 μM) induced a concentration-dependent decrease in heart rate, coronary flow, aortic flow, left ventricular developed pressure and its first derivative, dP/dt_{max} in isolated working rat heart. The effect of 10 nM capsaicin was mimicked by 0.1 nM endothelin. PD142893 (200 nM), a non-selective endothelin receptor blocking agent antagonized the effect of either endothelin (0.1 nM) or capsaicin (10 nM). We conclude that the majority of the effects of capsaicin in the rat heart are mediated by neural endothelin release.

Keywords: Capsaicin; Heart, isolated, working; Endothelin

1. Introduction

Stimulation of primary sensory nerves of the heart by capsaicin results in an increase in heart rate and coronary flow in isolated guinea-pig hearts. Regarding the neurotransmitters involved, the first evidence favoured a role for calcitonin gene-related peptide (CGRP) (Franco-Cereceda, 1988). Beyond CGRP, nitric oxide (NO) has also been shown to contribute to sensory nerve-mediated regulation of coronary flow and cardiac function, moreover the integrity of both transmitter systems has been suggested to be a prerequisite for the ability of the heart to adapt to repetitive ischaemic challenges in isolated working rat hearts (Ferdinandy et al., 1997). Nevertheless, the pharmacological effects of capsaicin in isolated heart of the rat have not been investigated (Szolcsanyi, 1996). The aim of the present work was therefore to study the effects of capsaicin on heart rate and cardiac function in isolated working rat heart.

2. Materials and methods

2.1. Ethics

The experiments performed in the present work conformed to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied was approved by the Ethical Committee of the University Medical School of Pécs.

2.2. Study design

Hearts from male Wistar rats (320–350 g) were prepared for working heart preparations perfused with temperature- (37°C) and pH- (7.2) controlled oxygenized Krebs–Henseleit bicarbonate buffer as described (Tosaki and Hellegouarch, 1994). After 10-min aerobic working perfusion, the hearts underwent various experimental protocols as follows.

Six hearts were exposed to increasing concentrations of capsaicin at log increments (1 nM–1 μM). Heart rate, coronary flow, aortic flow were monitored. Left ventricular developed pressure and its first derivative, dP/dt_{max} were also determined. The selected concentration (10 nM: EC_{50}) was then used for further studies. Since capsaicin decreased heart rate, coronary flow and deteriorated cardiac function, opposite to that would have been expected from studies with guinea-pig, we hypothesized the involve-

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ment of sensory neural endothelin (Giaid et al., 1989) immunolocalised in perivascular nerve fibres (Loesch et al., 1998). Therefore, the effect of 0.1 nM endothelin (separate group of 6 hearts) was also analyzed on these variables. In the last series of experiments, 10 nM capsaicin ($n = 6$) or 0.1 nM endothelin ($n = 6$) was co-perfused with 200 nM PD142893, a non-selective endothelin receptor antagonist (Brunner and Opie, 1998). The control for these series of experiments served those hearts exposed to 10 nM capsaicin or 0.1 nM endothelin alone ($n = 6$). Four hearts were given 200 nM PD142893 to study the per se cardiac effects of the endothelin antagonists. Five hearts were excluded from the study due to development of arrhythmias in response to high concentrations of capsaicin, so that altogether 34 hearts entered the whole study.

2.3. Drugs and chemicals

All drugs and chemicals were purchased from Sigma (St. Louis, Mo).

2.4. Statistical analysis

The data expressed as means ± standard error of means (S.E.M.) were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's t-test (Wallenstein et al., 1980). Changes were considered significant at $P < 0.05$.

3. Results

Capsaicin induced a concentration-dependent decrease in heart rate, coronary flow, aortic flow, left ventricular developed pressure and its first derivative, dP/dt_{max} (Table 1). The decrease in these parameters by the selected concentrations of capsaicin (10 nM) or endothelin (0.1 nM) was similar in magnitude. PD142893 (200 nM), a non-selective endothelin receptor blocking agent equally antagonized the effects of the selected concentrations of either endothelin or capsaicin (Fig. 1). It is worthy of note that the capsaicin-evoked responses were long-lasting after washout similarly as in the case of endothelin, i.e., the effects persisted 15 min subsequent to termination of the washout periods.

PD 142893 at the concentration applied was without effect on any of the baseline parameters studied.

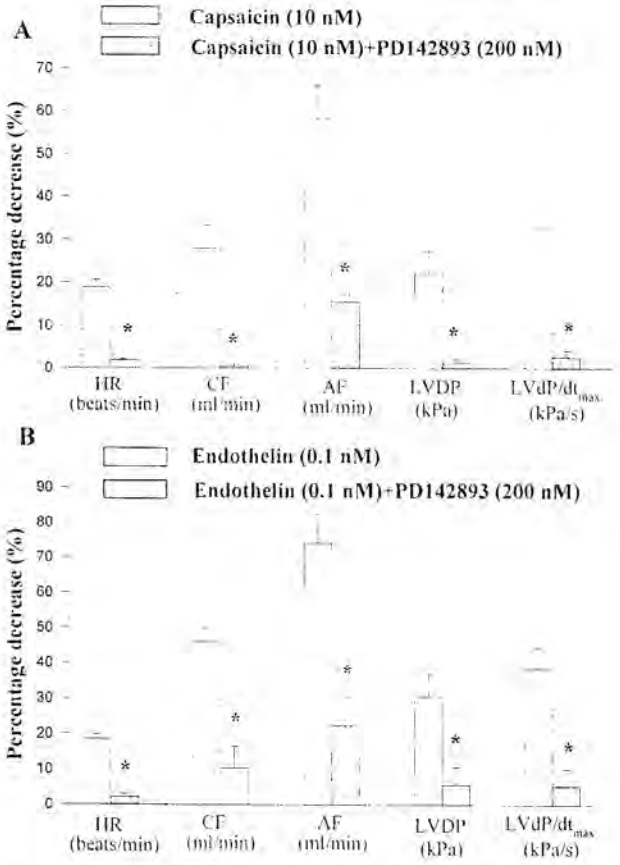

Fig. 1. Effect of capsaicin (A) or endothelin (B) on heart rate (HR), coronary flow (CF), aortic flow (AF), left ventricular developed pressure (LVDP), and its first derivative ($LVdP/dt_{max}$) in isolated working rat heart and an interaction with PD142893, an endothelin receptor antagonist. The data are means ± S.E.M. obtained with 6 preparations per group. * Indicates a significant difference between "capsaicin" vs. "capsaicin + PD142893" (A), and "endothelin" vs. "endothelin + PD142893" (B) values at $P < 0.05$.

Table 1
Effects of capsaicin on cardiac function
Data are expressed as means ± S.E.M. obtained with 6 hearts exposed to increasing concentrations of capsaicin at log increments in each group. Comparisons were made to the corresponding drug-free values. Abbreviations: HR (heart rate), CF (coronary flow), AF (aortic flow), LVDP (left ventricular developed pressure), $LVdP/dt_{max}$ (first derivative of LVDP).

Concentrations of capsaicin (nM)	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVDP (kPa)	$LVdP/dt_{max}$ (kPa/s)
0	318 ± 7	28.2 ± 3	58.8 ± 2.9	18.1 ± 0.1	801 ± 8.3
1	315 ± 8	27.0 ± 0.8	48.0 ± 3.0[a]	17.6 ± 0.3	767 ± 23
10	262 ± 10[a]	17.6 ± 1.5[a]	23.3 ± 3.6[a]	14.1 ± 0.9[a]	539 ± 35[a]
100	181 ± 13[a]	14.5 ± 2.1[a]	12.4 ± 4.7[a]	12.1 ± 1.0[a]	469 ± 29[a]
1000	92 ± 9[a]	7.0 ± 3.2[a]	3.6 ± 2.0[a]	2.6 ± 1.0[a]	97 ± 28[a]

[a] $P < 0.05$.

4. Discussion

The results show that exposure of the isolated working rat heart to capsaicin results in a progressive deterioration of cardiac function. It is also suggested that this effect is underlain by a secondary endothelin release, since (i) endothelin mimics the effects of capsaicin and (ii) PD142893 a non-selective endothelin receptor antagonist almost abolishes the aforementioned capsaicin effects.

Capsaicin-sensitive nerve endings serve as powerful peptidergic effector sites in several tissues (Szolcsanyi, 1996) including the heart (Franco-Cereceda, 1988) but the involvement of endothelin in tissue responses attributed to influences coming from these nerve endings has not been identified (Maggi, 1995). Sensory nerve fibres operating with NO and CGRP mediators in the rat heart have been found essential for the ability of the heart to tolerate ischaemia (Ferdinandy et al., 1997). In the guinea-pig, Franco-Cereceda (1988) described a decrease in coronary resistance and positive inotropy attributed to the release of CGRP from C fibres in guinea-pig heart. Surprisingly, in the rat direct stimulation of the heart by capsaicin attained a marked and abrupt decline in cardiac function possibly deriving from a conspicuous decrease in coronary flow, a finding contrary to that would have been expected from the effects of either CGRP or NO. The controversy at least in part is explained by a difference in the species, and differences in the process of sensory neurotransmitter release in response to myocardial ischaemia and that pharmacologically provoked by capsaicin in the non-ischaemic/normoxic heart. Similarly, a contradiction exists between results obtained by Sigrits et al. (1986), who found an increased rate and force of contraction by capsaicin using spontaneously beating isolated rat atria and those presented in our work. In the intact heart, coronary vasoconstriction induced by capsaicin is of dominant influence on cardiac contractility, whereas in isolated atrial preparations any effect on coronary tone has minimum or no effect on inotropy.

PD 142893 at the concentration applied blocked but did not reverse the effect of capsaicin on either coronary flow or heart rate, similar to other indicators of cardiac function although the concomitant release of CGRP, NO or substance P would have been expected to be responsible for an opposite effect. This may be explained by either an incomplete blockade of endothelin receptors by the antagonist with a substantial *residual* endothelin effect that masked the weak positive cardiac effects of the other sensory mediators or the lack of sufficient amounts of these latter substances in response to the capsaicin concentration applied in the intact heart. Moreover, it is important that the blocking effect with lack of reversal was seen at selected concentrations of capsaicin and the endothelin receptor antagonist, leaving the possibility that the spectrum of sensory neuropeptides released in response to higher capsaicin concentrations might be different with a more significant proportion of CGRP or substance P that might eventually be able to overcome the negative effects of endothelin.

As far as the mechanism of action of capsaicin in the heart is concerned, capsaicin opens cation channels gated by vanilloid receptors in a subset of sensory nerve terminals (Szolcsanyi, 1996). According to the best of our knowledge, sensory neurons are exclusive for vanilloid receptor expression (Caterina et al., 1997), thus, endothelin is probably released by capsaicin from the perivascular nerve fibres (Loesch et al., 1998; Giaid et al., 1989; Franco-Cereceda et al., 1991) and produces a marked coronary vasoconstriction with ensuing deterioration of cardiac function. However, the present study does not exclude the possibility of implication of vascular endothelium or endocardium as sources of endothelin. Nevertheless, neither endothelial cells nor cardiac myocytes express vanilloid receptors (Caterina et al., 1997); therefore, the endothelin releasing effect of capsaicin at least in the heart is possibly confined to sensory nerve fibres.

In summary, the study provides the first pharmacological evidence for the involvement of endothelin as new sensory neuropeptide underlying the effector function of cardiac capsaicin-sensitive sensory nerves.

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Functional and biochemical evidence for capsaicin-induced neural endothelin release in isolated working rat heart

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Abstract

In isolated working rat heart, capsaicin elicited a concentration-dependent constriction of coronary arteries accompanied by decline of all cardiac parameters recorded (heart rate, coronary and aortic flow, left ventricular developed pressure, and first derivative of left ventricular developed pressure). The following evidence suggests that capsaicin-induced changes are mediated by endothelin of neural origin: 1. Capsaicin (10 nM) evoked decrease in coronary flow resulting in deterioration of cardiac functions was mimicked by endothelin (0.1 nM). 2. The selective endothelin A receptor antagonist BQ-123 (1 μM) abolished the cardiac effects provoked by capsaicin (10 nM). 3. Reduction of extracellular calcium concentration from 2.4 to 1.2 or 0.6 mM inhibited the cardiac effects of capsaicin (10 nM) but not those induced endothelin (0.1 nM). 4. Perfusion the heart with 0.1 % (v/v) Triton X-100 damaged the endothelium and reversed the enhancement of coronary flow evoked by betanechol (1 μM), decreased the basal flow, but was without effect on capsaicin-induced coronary constriction. 5. Endothelin concentration measured in coronary effluent by radioimmunoassay increased up to 7-fold in response to capsaicin challenge (10-100 nM).

It is concluded, that in the rat heart, capsaicin acting on VR1 capsaicin receptors elicits a release of endothelin from the sensory nerve terminals.

Keywords: Capsaicin; Endothelin; BQ-123; Cardiac function; Isolated rat heart; Sensory neuropeptide

1. Introduction

Capsaicin, the pungent principle of red pepper due to its selective site of action on a subset of primary afferent neurons (Holzer, 1991; Szolcsányi, 1993; Maggi, 1995; Caterina et al., 1997) has become a valuable tool to reveal the function of the afferents. Activation of capsaicin-sensitive nociceptive, heat-sensitive and chemoceptive nerve endings (Szolcsányi, 1993) results in release of a variety of sensory neuropeptides, particularly tachykinins and calcitonin gene-related peptide (CGRP) which elicit various local tissue responses (Holzer, 1991; Maggi, 1995; Lundberg, 1996; Szolcsányi, 1996). The myocardium and coronary vessels are richly innervated by sensory neuropeptide containing primary afferent neurons sensitive to capsaicin (Lundberg et al., 1985; Saito et al., 1986; Wharton et al., 1986; Maggi, 1995; Lundberg, 1996). Although the neuropeptide releasing effects of capsaicin have been studied and well characterised in a wide variety of tissues, our knowledge concerning the capsaicin-elicited responses in the heart and coronary vessels is relatively sparse. The acute effects of capsaicin have been analysed in detail on the guinea-pig isolated perfused heart (Franco-Cereceda, 1988; Franco-Cereceda and Lundberg, 1985; Franco-Cereceda et al., 1991a; Oroszi et al., 1999) or on guinea-pig isolated atria (Fukuda and Fujiwara, 1969; Molnár et al., 1969; Franco-Cereceda, 1988; Maggi, 1995; Lundberg, 1996). Surprisingly, regarding rat heart, few data are available for the acute effects of capsaicin although studies on isolated perfused heart obtained from capsaicin-pretreated rats raised the issue of an important adaptive role in cardioprotection for CGRP and NO released from capsaicin-sensitive afferents (Ferdinandy et al., 1997; Song et al., 1999). In the rat, positive inotropic and chronotropic effects of capsaicin was described in the isolated atrial preparation

similar to that seen in hearts from the guinea-pig and have been attributed to the release of sensory neuropeptide CGRP (Franco-Cereceda, 1988; Maggi, 1995; Lundberg, 1996). Recently, however, we observed that capsaicin applied to the isolated working rat heart induced a decrease in coronary and aortic flow with negative inotropic effects resembling to the action of endothelin (Szolcsányi et al., 1999). The aim of the present paper is to shed light on the mechanism of capsaicin action on the isolated working rat heart. Particular emphasis was put on providing evidence for the hypothesis that capsaicin induced a release of endothelin from sensory nerve endings. It seemed to be important since in spite of some histochemical data indicating the existence of endothelin in perivascular nerve fibres (Giad et al., 1989; Franco-Cereceda et al., 1991b; Loesch et al., 1998) the presence of endothelin in capsaicin-sensitive sensory neurons or its release from sensory nerve endings in general has not been shown to date (Holzer, 1991; Maggi, 1995; Szolcsányi, 1996; Lundberg, 1996).

2. Methods

2.1. Animals

Male Sprague-Dawley rats (320-350 g body weight) were used for the experiments. The experimental protocols applied conformed to the European Community guiding principles for the care and use of laboratory animals and were approved by the local ethical committee of the University of Pecs, Hungary.

2.2 Isolated working heart preparation

Rats were anaesthetised with diethyl ether and then intravenous heparin (500 IU/kg) was given. After thoracotomy, the heart was excised and placed in ice-cold perfusion buffer. Immediately after preparation, the aorta and pulmonary vein were cannulated, and the heart was perfused (at 37 °C) according to the Langendorff method for a 5-min washout period at a constant perfusion pressure equivalent to 100 cm of water (10 kPa). The perfusion medium consisted of a modified Krebs-Henseleit bicarbonate solution: sodium chloride 118, potassium chloride 4.7, calcium chloride 2.4, sodium bicarbonate 25, potassium biphosphate 0.36, magnesium sulphate 1.2 and glucose 10 (mM). The Langendorff preparation was switched to the working mode following the washout period as previously described in detail by Tosaki and Braquet (1990), and Tosaki and Hellegouarch (1994). Aortic flow was measured by a calibrated rotameter. Coronary flow rate was measured by a timed collection of the coronary effluent that dripped from the heart.

Throughout all experiments heart rate (HR), coronary flow (CF), and aortic flow (AF) rates were registered. Left ventricular pressure (LVDP), and the first derivative of LVDP ($LVdP/dt_{max}$) were recorded by the insertion of a Millar catheter into the left ventricle via the left atrium and mitral valve.

2.3. Experimental protocols

In the first series of studies, extracellular calcium concentration was reduced from its control value of 2.4 to 1.2 or 0.6 mM. These extracellular calcium reductions were maintained during the

perfusion of 10 nM of capsaicin (selected according to the capsaicin dose-response curve, Szolcsanyi et al., 1999). Cardiac function was measured before (control), and after the perfusion of reduced calcium concentrations with capsaicin.

In the second series of experiments, 0.1 nM endothelin (Brunner and Opie, 1998) was perfused for 5 min and cardiac function was registered.

In the third series of the study, 0.1 nM endothelin was perfused in the presence of 0.6 mM of extracellular calcium for 5 min, and cardiac function was monitored.

In the next setting, 10 nM capsaicin was co-perfused with 1 μM of BQ-123 (Wang et al., 1998; Delpech et al., 1997), a selective endothelin A receptor antagonist for five minutes and cardiac function was measured.

In the following protocol, 0.1 nM of endothelin was co-perfused with 1 μM of BQ-123.

In the sixth set of experiments, 0.1 % (v/v) Triton X-100 (0.2 ml) was applied over 5 s into the coronary vasculature. This was succeeded by an immediate washout period according to aerobic Langendorff perfusion. Transient exposure to Triton X-100 served as a conventional method to destroy functional vascular endothelium in the intact heart (Kamata et al., 1996). The effect of capsaicin was then studied following switch to working mode.

2. 4. Measurement of endothelin

Coronary effluents (100 ml) were collected in polystyrene containers spiked with ethylene diamine tetra-acetic acid disodium salt (EDTA) and Triton X-100 to give final concentrations of 5 mM and 0.5 % (v/v), respectively and frozen -70 °C until they were processed. The perfusate was

loaded onto 3 ml methanol- and 5 ml water-conditioned Sep-Pak C_{18} cartridges (Waters), and endothelin was eluted with 2 ml 60 % (v/v) acetonitrile in 0.1 % (v/v) trifluoroacetic acid yielding a mean recovery of 60 %. The elutes were freeze-dried and kept at -20 °C until further radioimmunoassay determinations. Since the residue was re-dissolved in 0.5 ml of assay buffer, the coronary effluent samples were concentrated 200 times. Endothelin (i.e. endothelin-1, -2, and -3) immunoreactivity was then determined by means of radioimmunoassay using commercial endothelin radioimmunoassay kits (RIK 6910, Peninsula, Belmont, CA, USA) with cross-reactivity for endothelin 2: (142 %), endothelin 3 (98 %), big endothelin 1-38, big endothelin fragments 22-38, (<1 %); with inter-assay and intra-assay variations of below 10 % and 5 %, respectively (see for details: commercial kit descriptions).

2.5. Drugs and chemicals

Diethyl ether, Triton X-100 and Tween 80 were purchased from Reanal (Budapest, Hungary), betanechol (acetyl-beta-methylcholine) from Schuchardt (Munchen, Germany), heparin from Richter (Budapest, Hungary), capsaicin (8-methyl-N-vanillyl-6-nonenamide) and endothelin-1 from Sigma (St. Louis, USA), all constituents of the modified Krebs-Henseleit solution from Merck (Darmstadt, Germany). The endothelin radioimmunoassay kits were from Peninsula (Belmont, CA, USA). Capsaicin was dissolved in a solution consisting of 10 % (v/v) ethanol, 10 % (v/v) Tween 80 and 80 % (v/v) saline, then this stock solution was diluted with saline to the concentrations applied.

2.6. Exclusion criteria

Pre-selected exclusion criteria for the present studies demanded that hearts were excluded if: (1) ventricular arrhythmias occurred during the period prior to drug perfusions, (2) coronary flow and aortic flow were less than 19 and 35 ml/min, respectively, during drug-free aerobic perfusion. For the aforementioned reasons, 8 hearts were excluded from the entire study (75 hearts).

2.7. Statistical analysis

The data for myocardial function (HR, CF, AF, LVDP, and $LVdP/dt_{max}$) were expressed as the mean ± SEM. One-way analysis of variance was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of the drug-treated groups were compared with those of the drug-free control group by a modified t-test. A change of $P<0.05$ was considered significant.

3. Results

3.1. Effects of reduced extracellular calcium concentration on cardiac responses evoked by capsaicin or endothelin

In these studies, extracellular calcium concentration was reduced from its control value of 2.4 to 1.2 or 0.6 mM in the presence of 10 nM capsaicin. Capsaicin concentration was selected

according to the dose-response curve studied in the previous experiments. These alterations in extracellular calcium concentrations did not produce any significant change in baseline HR, CF, AF, LVDP, and $LVdP/dt_{max}$ (data not shown). The effects of capsaicin (10 nM) were markedly inhibited in the presence of reduced calcium (Table 1). Thus, at 2.4 mM calcium, coronary flow was reduced from its control value of 28.3 ± 0.3 to 18.2 ± 1.5 ml/min in the presence of 10 nM capsaicin. When capsaicin was co-perfused with 1.2 mM of calcium, the capsaicin-induced vasoconstriction was completely blocked as indicated by the non-significant reduction in coronary flow (26.9 ± 1.5 vs. 28.3 ± 0.3 ml/min). Similar non-significant reduction of the coronary flow was observed in the presence of 0.6 mM calcium. The reduction in extracellular calcium prevented the capsaicin-induced diminution in HR, AF, LVDP, and $LVdP/dt_{max}$ as well (Table 1).

Perfusion of the heart with 0.1 nM of endothelin-1 elicited a similar inhibition in HR, CF, AF, LVDP, and $LVdP/dt_{max}$ to that seen with capsaicin at a concentration of 10 nM (Table 1). At low calcium concentration (0.6 mM), the endothelin-induced responses were not prevented in contrast to the effects of capsaicin which was markedly inhibited at this calcium concentration (Table 1).

3.2. Impact of BQ-123, a selective endothelin A receptor antagonist on changes of cardiac function evoked by capsaicin or endothelin

Table 2 shows that BQ-123, a selective endothelin A receptor antagonist, suspended the effect of both capsaicin and endothelin suggesting that the capsaicin-induced responses were due to a release of endogenous endothelin in isolated working rat hearts.

3.3. Interaction between capsaicin and removal of functional endothelium by Triton X-100

Betanechol at concentration of 1 μM induced an increase in coronary flow, a response reversed by a preceding 0.1 % (v/v) Triton X-100 challenge over 5 s (Fig. 1a). Capsaicin (100 nM), however, elicited a decrease in coronary flow irrespective of the Triton X-100 pre-treatment (Fig. 1b). Note, that in both cases destruction of the coronary endothelium with Triton X-100 markedly decreased the basal coronary flow.

3.4. Effect of capsaicin on endothelin release

Endothelin concentration of the coronary effluent increased two-fold and seven-fold compared to the baseline in response to 10 nM and 100 nM capsaicin, respectively (Fig. 2).

4. Discussion

The present findings confirm our previous results (Szolcsanyi et al., 1999) that capsaicin at nanomolar concentrations markedly diminishes coronary flow resulting in deterioration of all cardiac functions in isolated working rat heart. The following experimental data support the concept that the effects observed are due to release of endothelin. 1. Direct radioimmunoassay determination revealed that capsaicin perfusion at low concentrations (10-100 nM) increased the endothelin level in the coronary effluent of the preparations up to seven-fold of the baseline

value. 2. Exogenous endothelin (0.1 nM) mimicked the effect of capsaicin (10 nM) inducing a similar inhibition with respect to heart rate coronary and aortic flow, left ventricular developed pressure and its first derivative (Klemm et al., 1995; Brunner et al., 1997; Geller et al., 1998; Tanak et al., 1997). In both cases the changes were long-lasting, practically irreversible. 3. The cardiac effects of both capsaicin and endothelin were antagonised by BQ-123, a selective endothelin A receptor blocking agent (Wang et al., 1998; Delpech et al., 1997). This finding is consistent with the results of our earlier experiments in which the non-selective endothelin antagonist PD142893 was applied (Szolcsányi et al., 1999).

Genes that encode the endothelins are expressed in a wide array of cells, although the major source of endothelin is thought to be of endothelial origin (Rubányi and Polokoff, 1994; Opgenorth, 1995; Miyauchi and Masaki, 1999). On the other hand, messenger RNA for endothelin-1 as well as the peptide itself have been localised in the dorsal root ganglia, where they co-exist with substance P and calcitonin gene-related peptide (Giad et al., 1989). Endothelin-like immunoreactivity (ET-LI) has also been shown in perivascular nerves of the rat basilar artery (Loesch et al., 1998). Furthermore, the majority of cultured dorsal root ganglion cells of the guinea-pig is ET-LI positive and the tissue content of ET-LI in dorsal root ganglia is several-fold higher than that of the stellate ganglion, spinal cord, heart, pulmonary artery or aorta being in fact the highest among the large scale of tissues investigated (Franco-Cereceda et al., 1991b).

Selective site of action of capsaicin on a subset of polymodal type nociceptors with C- and A-delta fibres was described by single unit studies not only in cutaneous nerves but also in nerve branches which innervate the heart and great vessels (Holzer, 1991; Szolcsányi, 1993,

1996; Maggi, 1995). Moreover, the recently cloned capsaicin receptor denoted as VR-1 receptor was expressed in the small type sensory neurons but was undetectable in the heart tissue of the rat (Caterina et al., 1997). Taking together, all these findings suggest that sensory nerve terminals and not the endothel of the coronary vessels or the heart are the source of endothelin released.

In the present study, two lines of evidence support this conclusion. 1. Removal of functional endothelial cells by Triton X-100 (Kamata et al., 1996) was incapable of preventing capsaicin-induced decrease of the coronary flow and its deleterious effects on cardiac functions. On the other hand, the pre-treatment reversed vasodilatory effect of the cholinergic agonist betanechol and also decreased basal coronary flow indicating that the Triton X-100 treatment used was appropriate to destroy the functional endothelium. 2. The other approach exploited the fact that capsaicin elicits a calcium-dependent release of sensory neuropeptides which has been analysed in detail on substance P and CGRP (Holzer, 1991; Maggi, 1995). Here we have shown that the pronounced coronary and cardiac effects of capsaicin were inhibited and abolished by reducing the extracellular calcium concentration from its control value of 2.4 to 1.2 or 0.6 mM, respectively. Inhibitory effect of reduced calcium concentration in the perfusate cannot be attributed to a diminished responsiveness of the coronary vessels or cardiac muscle to the endothelin released since the effect of exogenous endothelin remained practically unchanged under these conditions. It is relevant, that in isolated perfused rat heart subjected to 15-min ischaemia by coronary occlusion, subsequent perfusion with 1 μM capsaicin did not affect either coronary flow or heart rate, whereas in non-ischaemic rat hearts it reduced left ventricular developed pressure and coronary flow (D'Alonzo et al., 1995). It is tempting to assume, that the

absence of the capsaicin response after ischaemia is an indicator of neural damage.

The deleterious effect of capsaicin on cardiac function of the rat working heart is probably due to its pronounced inhibitory effect on coronary flow and oxygen supply. This conclusion is supported by the fact that capsaicin, as well as its putative sensory neuropeptide mediators of CGRP and endothelin displayed positive inotropic and chronotropic effects in the isolated atrial preparations of the rat or guinea-pig (Fukuda and Fujiwara, 1969; Molnár et al., 1969; Franco-Cereceda, 1988; Lembeck et al., 1989). It is intriguing, however, that perfusion the guinea-pig Langendorff heart preparation with 1 μM capsaicin produced no ET-LI release in the outflow albeit ET-LI in sensory neurons is high in this species (Franco-Cereceda et al., 1991b). The absence of evidence for the release of endothelin from guinea-pig hearts and the relative insensitivity of the guinea-pig coronary arteries to endothelin (as compared to that of the rat, Lembeck et al., 1989) might explain why capsaicin enhances and does not diminish the coronary flow in this species. It is remarkable, that simultaneous detection of CGRP released and capsaicin-induced changes in frequency, tension and perfusion volume in the Langendorff-perfused guinea-pig heart preparation showed that 100 nM capsaicin induced a more than tenfold increase of the CGRP-LI in the outflow without evoking any significant change in the perfusion pressure or volume (Franco-Cereceda et al., 1991a). Consequently, in guinea-pig heart the primary sensory neuropeptide of functional significance is CGRP eliciting coronary vasodilatation and positive inotropic effects while in the rat heart capsaicin releases endothelin which probably owing to its potent and practically irreversible effects prevents the manifestation of the increased coronary flow and positive inotropic effect mediated by the release of CGRP or NO (Franco-Cereceda, 1988; Franco-Cereceda et al., 1991a; Ferdinandy et al., 1997).

In rats, systemic pre-treatment with high capsaicin doses several days before the experiment induces sensory blockade "desensitisation" with depletion of CGRP and NO from the cardiac sensory fibres (Ferdinandy et al., 1997). In these pre-treated rats impairment in cardiac protection elicited by pacing-induced preconditioning or heat stress (Ferdinandy et al., 1997; Song et al., 1999) was observed which might indicate that several days after capsaicin treatment the impairment in the release vasodilator mediators prevail over the presently described endothelin-mediated responses.

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LEGEND FOR FIGURES:

Figure 1. A. Endothelium-dependent vasorelaxation by betanechol in isolated Langendorff-perfused rat heart. B. Endothelium-independent vasoconstriction by capsaicin. Removal of functional coronary endothelium resulted from a preceding brief (5 s) exposure to 0.1 % (v/v) Triton X-100. Data are means±S.E.M. obtained with 6 preparations. *: significantly different from corresponding control at $P<0.05$.

Figure 2. Capsaicin-induced endothelin release. A concentration-response relationship. Endothelin (endothelin 1-, 2-, and 3) concentration was determined in coronary effluent of the Langendorff-perfused rat hearts by means of radioimmunoassay. The results are means± S.E.M. obtained from 6 determinations with 6 separate preparations. *: significantly different from baseline at $P<0.05$.

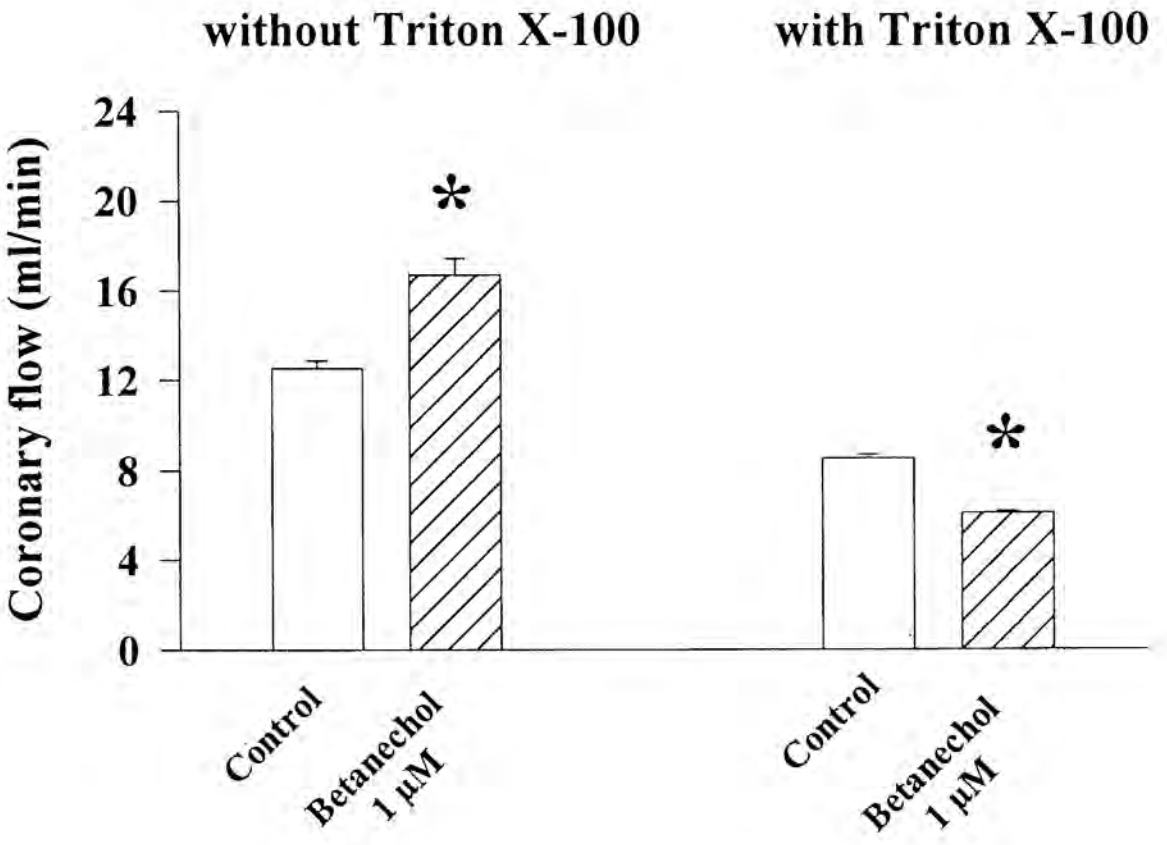
Table 1. Effects of capsaicin and endothelin on cardiac function in the presence of normal or reduced extracellular calcium concentration $[Ca^{2+}]_o$. Data are expressed as means ± S.E.M. obtained with 6 hearts in each group. Comparisons were made to the corresponding drug-free values (*$P<0.05$). Abbreviations: HR (heart rate), CF (coronary flow), AF (aortic flow), LVDP (left ventricular developed pressure), $LVdP/dt_{max}$ (first derivative of LVDP).

Concentration of substances	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVDP (kPa)	$LVdP/dt_{max}$(kPa/s)
2.4 mM $[Ca^{2+}]_o$ (control)	313 ± 9	28.3 ± 0.3	55.2 ± 2.4	18. ± 0.1	794 ± 9
2.4 mM $[Ca^{2+}]_o$ + Capsaicin 10 nM	254 ± 12 *	18.2 ± 1.5 *	24.2 ± 3.1 *	13.8 ± 0.7 *	527 ± 31 *
1.2 mM $[Ca^{2+}]_o$ + Capsaicin 10 nM	278 ± 11 *	26.9 ± 1.5	38.6 ± 1.9 *	16.9 ± 0.2	701 ± 12 *
0.6 mM $[Ca^{2+}]_o$ + Capsaicin 10 nM	308 ± 7	26.7 ± 2.1	46.5 ± 1.2 *	17.6 ± 0.1	766 ± 10
2.4 mM $[Ca^{2+}]_o$ + Endothelin 0.1 nM	253 ± 8 *	16.4 ± 1.3 *	18.2 ± 3.9 *	11.8 ± 1.2 *	489 ± 35 *
0.6 mM $[Ca^{2+}]_o$ + Endothelin 0.1 nM	248 ± 11 *	16.7 ± 2.19 *	22.8 ± 4.3 *	13.8 ± 0.9 *	543 ± 40 *

Table 2. Effects of capsaicin and endothelin on cardiac function in the presence or absence of BQ-123, a selective endothelin A receptor blocking agent. Data are expressed as means ± S.E.M. obtained with 6 hearts in each group. Comparisons were made to the corresponding drug-free values (*$P<0.05$). Abbreviations: HR (heart rate), CF (coronary flow), AF (aortic flow), LVDP (left ventricular developed pressure), $LVdP/dt_{max}$ (first derivative of LVDP).

Concentration of substances	HR (beats/min)	CF (ml/min)	AF (ml/min)	LVDP (kPa)	$LVdP/dt_{max}$(kPa/s)
Control	316.± 7	29.5 ± 1.68	60.7 ± 4.9	18.1 ± 0.1	808 ± 11.1
Capsaicin 10 nM	258 ± 11 *	16.9 ± 1.3 *	21.5 ± 2.8 *	14.2 ± 0.7 *	541 ± 39 *
Capsaicin 10 nM + BQ-123 1 μM	327 ± 8	30.8 ± 1.47	58.6 ± 4.1	17.3 ± 0.2	796 ± 15
Endothelin 0.1 nM	257 ± 9 *	17.4 ± 1.7 *	17.4 ± 5.3 *	12.6 ± 1.1 *	498 ± 48 *
Endothelin 0.1 nM + BQ-123 1 μM	311 ± 12	28.8 ± 1.2	56.1 ± 3.7	17.8 ± 0.1	785 ± 13

A


without Triton X-100
with Triton X-100
Coronary flow (ml/min)
24
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Control
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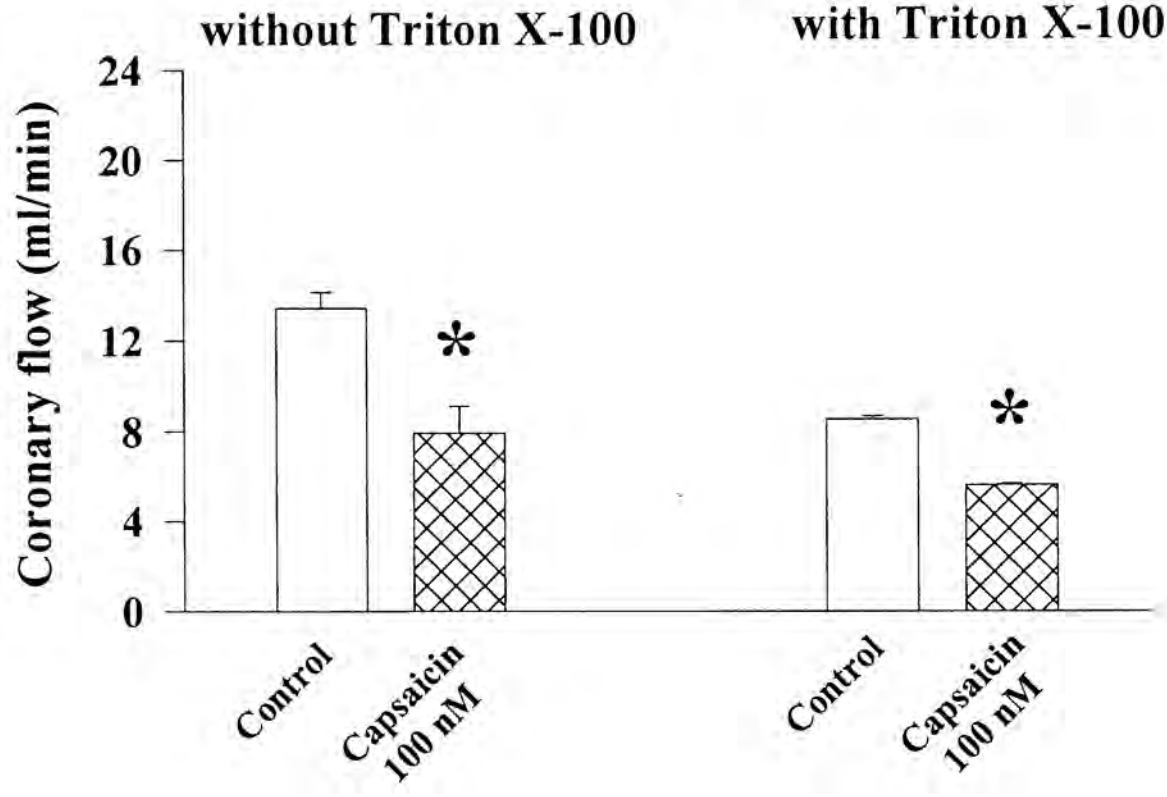
B


without Triton X-100
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Coronary flow (ml/min)
24
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Capsaicin 100 nM
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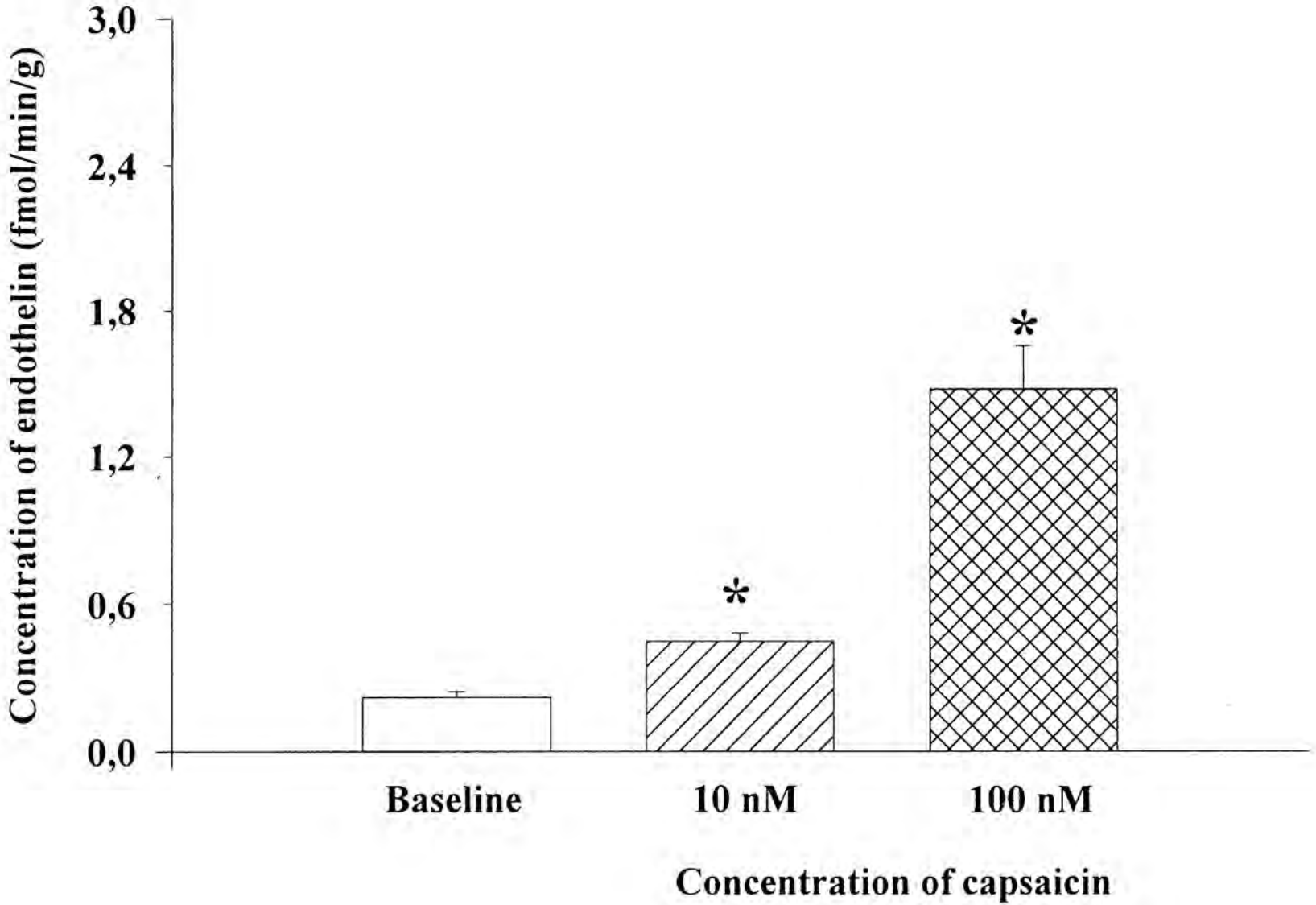
Capsaicin 100 nM


3,0
2,4
1,8
1,2
0,6
0,0
Concentration of endothelin (fmol/min/g)
*
*
Baseline
10 nM
100 nM
Concentration of capsaicin

Impaired capsaicin-induced decrease in heart rate and coronary flow in isolated heart of diabetic rats

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Abstract

We studied whether streptozotocin (50 mg/kg i.v.)-induced diabetes associated with sensory neuropathy modified the effect of capsaicin (0.1 μM) on heart rate and coronary flow in Langendorff-perfused rat heart. Baseline heart rate and coronary flow decreased from 317.9±2.9 b.p.m. and 13.4 ±0.7 ml/min to 255.1±12.7 and 219.8±2.8 b.p.m. and 8.9±0.6 and 10.0±0.1 ml/min ($P<0.05$) in hearts from animals with 4- and 8-week diabetes, respectively. Capsaicin significantly decreased both variables in either normal or 4-week diabetic animals, however, it failed to affect coronary flow or heart rate in preparations from 8-week diabetic rats. Endothelin-1 (0.1 nM) significantly decreased heart rate and coronary flow irrespective of the presence or absence of diabetes. The streptozotocin-treated animals exhibited a significant decrease in femoral nerve conduction velocity involving both fast conducting A- and slow-conducting C-fibres proportional to the duration of the pre-existing diabetic state. We conclude that the capsaicin-induced decrease in heart rate and coronary flow are attenuated in insulin deficient diabetes. This is related to a feeble endothelin release without changes in post-receptor mechanisms mediating the endothelin effects.

Keywords: Sensory nerve function; Capsaicin; Endothelin; Diabetes mellitus; Rat heart

1. Introduction

A decrease in the release of sensory neuropeptides such as calcitonin gene-related peptide (CGRP), substance P and somatostatin in response to electical stimuli is characteristic of diabetic neuropathy (Nemeth et al., 1999a). Recently, this has been suggested to underlie a deficiency in several adaptive mechanisms attributed to the integrity of sensory-effector function such as neurogenic inflammation (Walmsley and Wiles, 1991; Gyorfi et al., 1996, Nemeth et al., 1999b) and the ability of the heart to adapt to repetitive ischaemic insults i.e. the ischaemic preconditioning phenomenon (Tosaki et al., 1996; Ferdinandy et al., 1997).

Capsaicin stimulating vanilloid receptors located on a subpopulation of primary afferent fibres has been shown to decrease heart rate and coronary flow with an ensuing deterioration of cardiac function in isolated working rat hearts but an increase in heart rate with coronary vasodilation and positive inotropy by capsaicin were seen in isolated guinea-pig hearts. As far as the neurotransmitters involved is concerned, nitric oxide and CGRP have been shown to mediate the capsaicin-induced positive cardiac effects in guinea-pigs (Franco-Cereceda 1988; Oroszi et al, 1999a,b), however, neural endothelin is suspected to produce the negative effects of capsaicin in the rat heart (Szolcsanyi et al., 1999). Thus, we sought whether experimental diabetes associated with combined (sensory and motor) neuropathy induced by streptozotocin influenced the effects of capsaicin on isolated Langendorff-perfused rat heart.

2. Materials and methods

2.1. Ethics

All experimental protocols applied in the present study conformed to the Europian Community guiding principles for the care and use of laboratory animals and were approved by the local ethical committee of Medical University of Pecs, Hungary.

2.2. Experimental groups

The experiments were carried out with 96 male Wistar rats weighing 300-350 g. The animals were housed in the Laboratory Animal Center of the Medical University of Pecs under pathogen free conditions provided with standard rat chow and tap water ad libitum. The animals were divided into four groups (Fig.1). Group 1 and 3: rats were treated with the solvent for streptozotocin 4 or 8 weeks before the experiments. The animals in Group 2 and 4 were given streptozotocin (50 mg/kg i.v.) at the same time. Each group consisted of 24 rats, with subgroups of 12 animals for studies on isolated heart preparations, 6 rats for plasma insulin and glucose levels, and a subset of 6 animals was used for nerve conduction velocity measurements.

2.3. Determination of heart rate and coronary flow in Langendorff-perfused isolated rat hearts

It has been described in detail elsewhere (Tosaki et al., 1993). Briefly, 6 rats were anaesthesized with diethyl ether. Na-heparin (500 IU/kg i.v.) was given to prevent blood

coagulation. Following thoracotomy, the hearts were excised and mounted on a Langendorff apparatus. Following a 20 min period of aerobic perfusion with modified Krebs-Henseleit solution, the hearts were exposed to 0.1 μM capsaicin or 0.1 nM endothelin-1 for 5 min. Baseline values were determined at the end of the equilibration period, the drug-induced changes were determined immediately after capsaicin/endothelin-1 exposure. Both capsaicin and endothelin-1 exhibited a long-lasting effect, however, baseline values could be re-captured after a 2-hour washout period.

2.4. Assessement of plasma insulin and glucose concentration

Plasma insulin level was measured by radioimmunoassay (RIA). The glucose oxidase method was used for fasting blood glucose determination.

2.5. Nerve conduction velocity

We used the method described earlier (Nemeth et al., 1999b). In brief, the left femoral nerve was prepared and stimulated with trains of square-wave constant voltage stimuli (500 μs) applied through a pair of platinum electrodes. Another pair of electrodes was applied 2 cm distal to the stimulating electrodes for recording compound action potentials. The average time lags between stimulation signals and the appearance of corresponding „A" and „C" waves were determined. Dividing the distance between the stimulating and receiving electrodes by the interval between the stimulatory impulses (20 stimuli) and appearance of the corresponding „A" and „C" signals produced the data for evaluation (Janig and Lisney, 1989; Nemeth et al., 1999b).

2.6. Drugs and solutions

Sodium thiopentone (Trapanal) was purchased from Byk Gulden (Konstanz, Germany), streptozotocin (Zanosar) from Upjohn (Kalamazoo, USA), diethyl ether and Tween 80 from Reanal (Budapest, Hungary), heparin Richter (Budapest, Hungary), capsaicin (8-methyl-N-vanillyl-6-nonenamide) and endothelin-1 from Sigma (St. Louis, USA), insulin RIA kit from Izinta (Budapest, Hungary), all constituents of the modified Krebs-Henseleit solution from Merck (Darmstadt, Germany). Capsaicin was dissolved in a solution consisting of 10 % (v/v) ethanol, 10 % (v/v) Tween 80 and 80 % (v/v) saline.

2.7. Statistical analysis

The data are expressed as means±standard error of the mean (S.E.M.). Changes in heart rate and coronary flow were evaluated by means of analysis of variance (ANOVA) followed by a modified t-test for repeated measurements according to Bonferroni's method (Wallenstein et al., 1980). Plasma insulin, glucose and sciatic nerve conduction velocity values were analyzed with Student's t-test for unpaired data. Changes were considered significant at $P<0.05$.

3. Results

3.1. Effects of diabetes on body weight, fasting blood glucose and plasma insulin levels

The animals in Group 1 and 3 exhibited a continuous weight gain over the examination period, whereas the rats from Group 2 and 4 revealed a moderate weight loss. Plasma insulin

level significanly decreased with an ensuing increase in blood gucose level in the streptozotocin-treated groups at both 4 and 8 weeks after injection (Table 1).

3.2. Nerve conduction velocity test

Nerve conduction velocity test was used as a "gold standard" of verifying diabetic neuropathy. Streptozotocin produced a decrease in conduction velocity in fast conducting myelinated A-fibres: 28.1±1.29 m/s normal rats (Group 3) vs 15.3±1.66 and 10.9±1.21 m/s (P<0.05 for both) in 4-week and 8-week diabetic animals (Group 2 and 4), respectively. Slow conducting unmyelinated C-fibres disclosed a similar response. Conduction velocity was 0.64±0.09 m/s in normal animals (Group 3) and it decreased to 0.44±0.03 and 0.35±0.04 m/s (P<0.05 for both) in 4-week and 8-week diabetic rats (Group 2 and 4), respectively. The values of conduction velocity of fast and slow conducting fibres did not differ from each other in Group 1 and 3.

3.3. Effect of diabetes on changes in heart rate and coronary flow in response to capsaicin

Both heart rate and coronary flow significantly decreased in hearts from animals treated with streptozotocin (Group 2 and 4). Capsaicin (0.1 μM) significantly decreased both variables in either normal (Group 3) or 4-week streptozotocin-diabetic (Group 2) animals, however, capsaicin failed to affect coronary flow or heart rate in preparations from 8-week diabetic (Group 4) rats (Fig. 2A,B). Corresponding values obtained in hearts from the solvent treated groups (1 and 3) did not significantly differ from each other (data not shown).

3.4. Effect of endothelin on heart rate and coronary flow in isolated hearts from normal and diabetic rats

Endothelin-1 (0.1 nM) significantly decreased heart rate and coronary flow irrespective of the presence or absence of diabetes (Fig. 3A,B).

4. Discussion

These results are in accordance with previous findings that capsaicin decreases heart rate and coronary flow in isolated rat heart (Szolcsanyi et al., 1999). The effects of capsaicin, however, have been shown to result from a secondary sensory neurotransmitter release in concert with the finding that vanilloid receptors are exclusively expressed on sensory neurons (see for review Szolcsanyi, 1996, Caterina et al., 1997). Regarding the mediators involved in the rat heart, most evidence favoured a role for endothelin since PD 142893, a non-selective endothelin receptor antagonist abolished the effects of capsaicin (Szolcsanyi et al., 1999). This is also confirmed, since endothelin-1 produced effects on heart rate and coronary flow similar to those evoked by capsaicin. The original observation of the present work is that the cardiac effects of capsaicin impair with the development of diabetic neuropathy as indicated by a relatively preserved capsaicin effects at an early diabetic state (4 weeks) with a significant attenuation of capsaicin responses in 8-week diabetic animals exhibiting a significant degree of sensory neuropathy.

Diabetes is known to produce several pathological changes in the cardiocascular system including changes in blood vessel structure, endothelial function, heart metabolism and neural dysfunction involving motor, sensory and autonomic nerves (see for reviews Scheen, 1997; Paulson,1997; Stehouwer et al., 1997). As far as the mechanism of sensory dysfunction in

insulin-deficient diabetes is concerned, a defective axonal transport is thought to be the key initiating factor that eventually results in sensory nerve degeneration as a characteristic late complication of the disease (see for review, Fedele and Giugliano, 1997). Diabetic neuropathy leads to a functional damage of sensory-effector machinery characterized by a significant decrease in sensory neurotransmitter release such as that of somatostatin, substance P and calcitonin gene-relat peptide (CGRP) that precedes neurodegenerative structural changes (Nemeth et al., 1999a). Therefore, it is not surprising, that important cardiovascular adaptive mechanisms such as ischaemic preconditioning known to be triggered by a release of sensory neurotransmitters in response to metabolic changes in the ischaemic myocardium such as CGRP and nitric oxide (NO) (Ferdinandy et al., 1997) are seriously impaired in diabetes (Tosaki et al., 1996).

The principal sensory neurotransmitter released by capsaicin in isolated rat heart is endothelin (Szolcsanyi et al., 1999). According to the present results, while a marked attenuation of capsaicin-induced decrease in heart rate and coronary flow occurred, vasoconstrictive and frequency responses to endothelin were not significantly different between normal and diabetic animals, indicating that diabetes caused peripheral sensory neural dysfunction in the rat heart and not alterations of post-receptor signalling pathways for endothelin. Nevertheless, endothelin may act both on smooth muscle cells of the coronary arteries and on endothelial receptors mediating opposite vascular effects i.e. vasoconstriction by stimulation smooth muscle receptors and vasodilation by releasing endothelial vasorelaxants (Chokkukannan et al., 1998). It is clearly shown in (Fig. 2B and 3B) that baseline coronary flow is significantly decreased in diabetic hearts, thus, it is concievable to speculate that coronary vasoconstriction by endothelin may even be exaggerated in diabetes, however, in our experiments, it did not reach statistical significance. Irrespective of whether to what degree the impairment of endothelial fuction contributes to vasoconstrictive responses to either endothelin

or capsaicin in the diabetic rat heart, the results show that function of both cardiac sensory nerves and coronary endothelium is significantly impaired in steptozotocin diabetes of 8-week duration in rats. Besides a significant attenuation of cardiac capsaicin effects and a decrease in baseline coronary flow, the streptozotocin-treated rats had characteristic features of Type I diabetes mellitus in that they failed to gain weight and exhibited very low plasma insulin levels associated with hyperglycaemia. Moreover, nerve conduction velocity studies revealed sensory neuropathy proportional to the duration of diabetes.

The reduction of the capsaicin effects in diabetes are highly consistent with observations demonstrating a depletion of neurotransmitters (Diemel et al., 1992) and a decreased neuropeptide release from sensory nerves in response to a standardized challenge in rats with streptozotocin diabetes (Nemeth et al., 1999a) The rich sensory innervation of the heart raised the possibility that these nerves are fundamentally involved in development of the ischaemic preconditioning phenomenon (Ferdinandy et al., 1997; Tang et al., 1999; Zhou et al., 1999), the most powerful cardioprotective mechanism described to date (see for review Lawson and Downey, 1993). Epidemiological studies have clearly demonstrated that patient with either insulin-dependent diabetes mellitus or non-insulin-dependent diabetes mellitus are more prone to myocardial infarction and post-myocardial complications independ of whether or not they have coronary atherosclerosis (Stone et al., 1989). It has been known for a long time that diabetes is an independent risk factor for ischaemic heart disease, but there is much debate as to whether how the diabetic heart becomes more susceptable to ischaemic injury. A study in which the evolution of diabetes in relation to ischaemia-reperfusion injury was investigated, the diabetic rat heart was found more resistant to ischaemia-reperfusion-injury in the early phase of diabetes (2-week), but this additional protection was then lost after an additional 4-6-week diabetic period. The animals which had been diabetic for eight weeks, a worse outcome from ischaemia-reperfusion occured as compared to hearts from normal animals (Tosaki et al.,

1996). Our results closely correlate with these data, since responses to capsaicin were not significantly impaired in 4-week diabetic animals, however the capsaicin-induced changes were completely abolished after a pre-existing 8-week diabetic period. Taken together the role of sensory nerves in the adaptability of the heart to ischaemia and the impairment of these nerves in diabetes with the loss of the preconditioning phenomenon in advanced diabetic state, it seems suggestive that functional impairment of sensory nerve underlies the decrease in the capability of the heart to adapt to repetitive ischaemic insults in an advanced diabetic state.

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Legends for figures

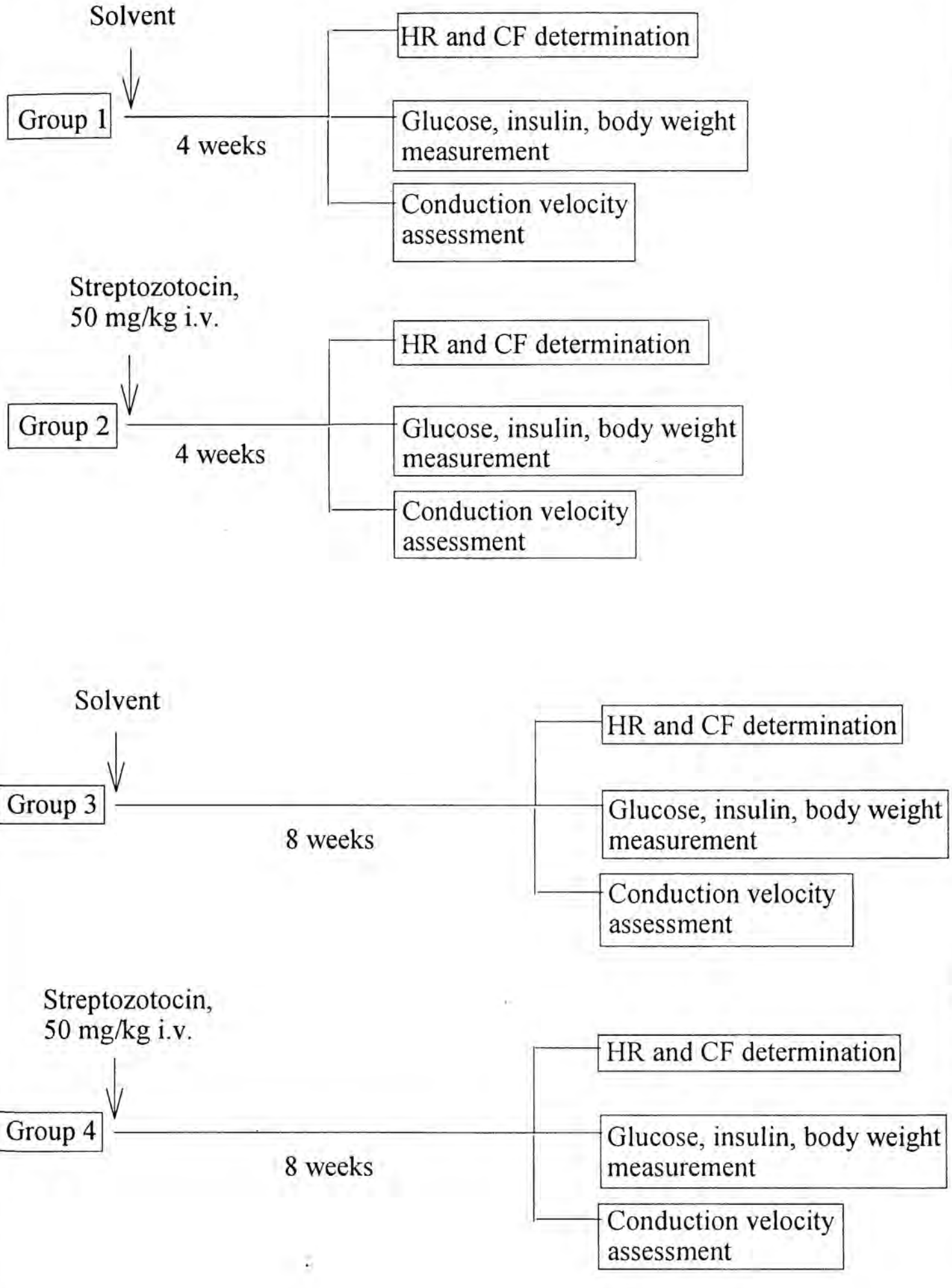
Fig. 1. Studies on the cardiac effects of capsaicin in rats with streptozotocin-induced diabetes. Study design. Abbreviations: HR: heart rate; CF: coronary flow (determinations in Langendorrf-preparations).

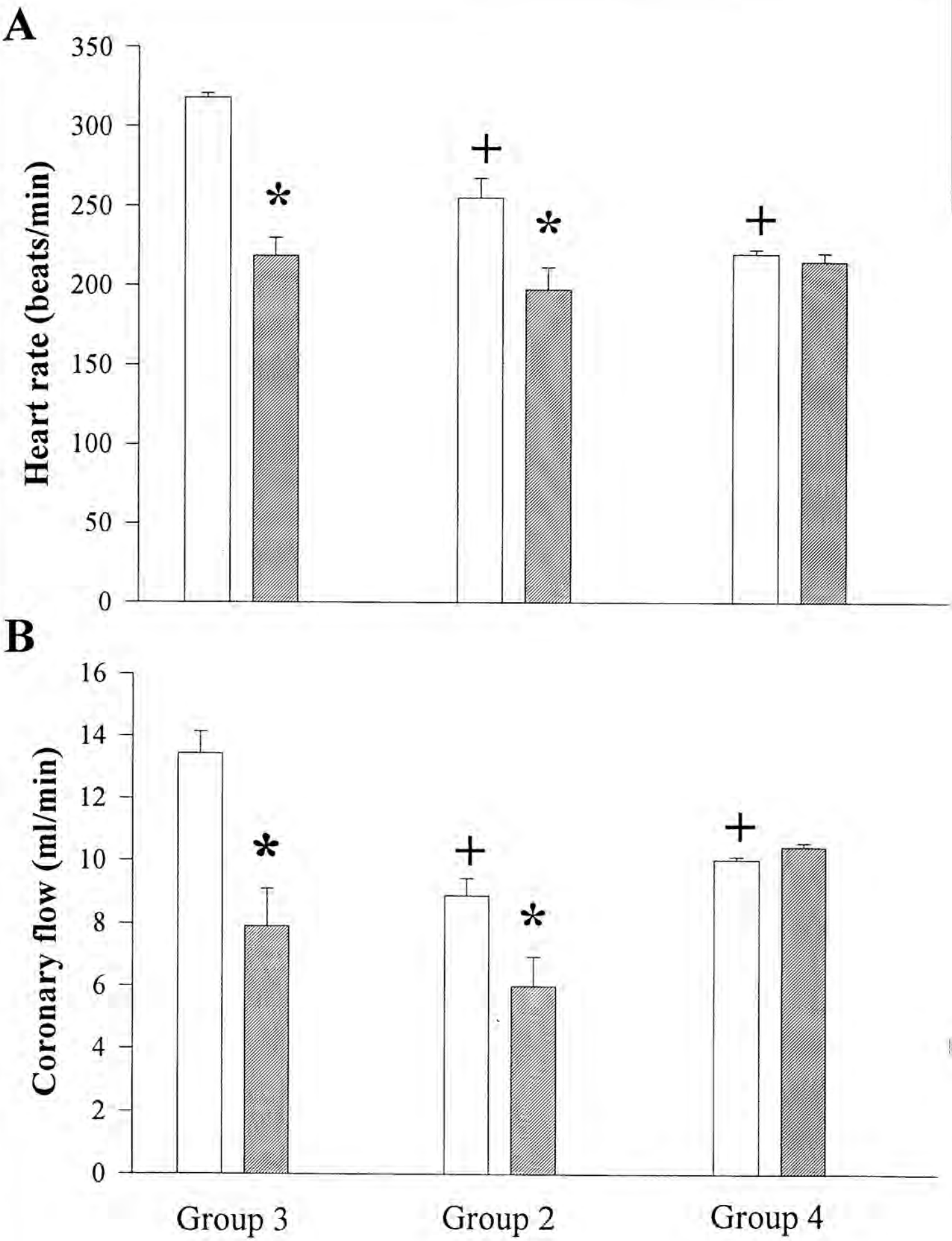
Fig.2. Changes in heart rate (A) and coronary flow (B) in isolated Langendorff-perfused rat hearts in response to capsaicin. The first pair of columns represents data obtained from solvent treated animals (Group 3), the second pair of columns denotes values from 4-week diabetic rats (Group 2) and the third one designates data from 8-week diabetic animals (Group 4). Data are means±S.E.M., * indicates significant difference between baseline values (blank columns) and those following capsaicin exposure (hatched columns). + shows significant changes between baseline values of Group 3 compared to those of Group 2 and 4 (blank columns). n=6 in each group. *$P<0.05$, +$P<0.05$.

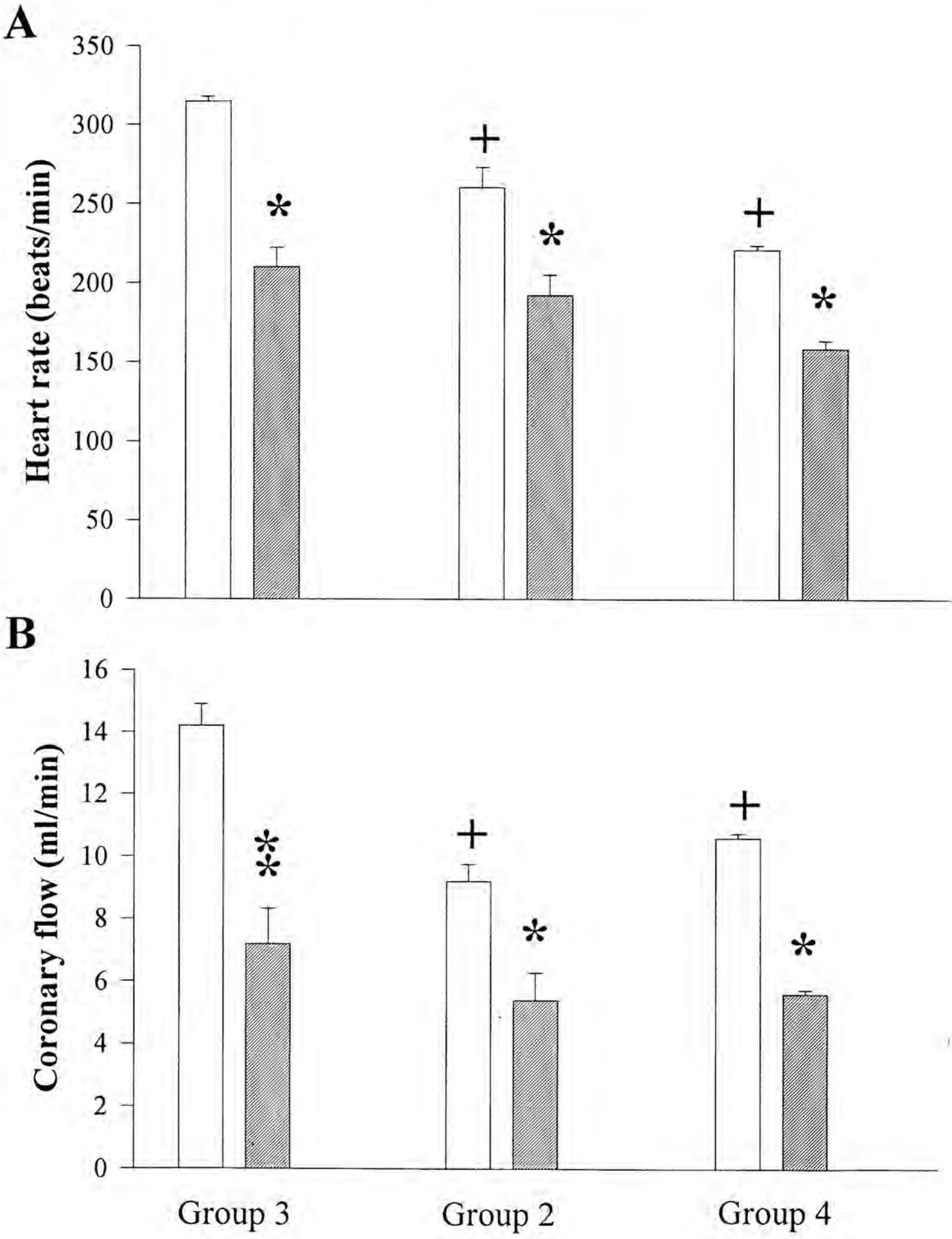
Fig.3. Heart rate (A) and coronary flow (B) alterations by endothelin in Langendorff-perfused rat hearts. The first pair of columns shows data obtained with solvent treated animals (Group 3), the second pair of columns represents results from 4-week diabetic rats (Group 2) and the third one denotes data from 8-week diabetic animals (Group 4). Data are means±S.E.M., * indicates significant difference between baseline values (blank columns) and those subsequent to endothelin exposure (hatched columns). + shows significant changes in baseline values of preparations from Group 3 compared to those from Group 2 and 4 (blank columns). n=6 in each group. *$P<0.05$, +$P<0.05$

Table 1 shows changes in body weight (+/- denotes weight gain/loss), values of fasting blood glucose and plasma insulin levels in 4-week or 8-week diabetic rats and in the correspoding controls. Data are expressed as means±S.E.M., *P<0.05, comparisons were made to the corresponding control groups.

	Group1	Group 2	Group3	Group4
Changes in body weight (g)	+69.5±3.9	-6.8±1.2	+112.4±6.5	-11.2±2.3
Fasting blood glucose (mmol/l)	5.8±0.2	18.1±2.2 *	6.1±0.3	21.1±3.1 *
Plasma insulin (μIU/ml)	10.3±2.5	2.6±0.5 *	10.5±2.8	2.7±0.8 *


Solvent
Group 1
4 weeks
HR and CF determination
Glucose, insulin, body weight measurement
Conduction velocity assessment
Streptozotocin, 50 mg/kg i.v.
Group 2
4 weeks
HR and CF determination
Glucose, insulin, body weight measurement
Conduction velocity assessment
Solvent
Group 3
8 weeks
HR and CF determination
Glucose, insulin, body weight measurement
Conduction velocity assessment
Streptozotocin, 50 mg/kg i.v.
Group 4
8 weeks
HR and CF determination
Glucose, insulin, body weight measurement
Conduction velocity assessment


A
Heart rate (beats/min)
350
300
250
200
150
100
50
0
*
+
*
+
B
Coronary flow (ml/min)
16
14
12
10
8
6
4
2
0
*
+
*
+
Group 3
Group 2
Group 4


A
Heart rate (beats/min)
350
300
250
200
150
100
50
0
+
*
+
*
+
*
B
Coronary flow (ml/min)
16
14
12
10
8
6
4
2
0
*
+
*
+
*
Group 3
Group 2
Group 4