Pharmacology of the human isolated coronary artery

Effects of 5-HT, platelets, and peptides

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EFFECTS OF 5-HT, PLATELETS, AND PEPTIDES

FARMACOLOGIE VAN DE GEÏSOLEERDE HUMANE CORONAIR ARTERIE

EFFECTEN VAN SEROTONINE, THROMBOCYTEN EN PEPTIDEN

PROEFSCHRIFT

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a vaillans cuers riens impossible

Jacques Coeur, ca. 1395-1456

Aan mijn vader en moeder
Aan mijn grootouders
Voor Colette

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Part 1

Introduction

Chapter 1

Clinical aspects of ischemic heart disease

1.1 Introduction

Ischemia of the heart is characterized by a deprivation of oxygen combined with inadequate removal of metabolites. Myocardial ischemia may be due to an increased oxygen requirement, or to a decreased supply of oxygen to the myocardium.

The myocardial consumption of oxygen is determined by myocardial wall tension, contractility, and heart rate. Processes in the myocardium, not directly related to contractile function, require only 20% of the total oxygen consumption, while the amount needed for electrical depolarization is only approximately 1% of total oxygen consumption [1, 2]. The myocardial oxygen demand is increased by physical exercise or by mental stress. In healthy individuals, an increased oxygen demand is likely to be compensated via an increase of coronary blood flow [3]. An increased oxygen demand may result in cardiac ischemia only in patients with existing coronary artery impairment [4].

Myocardial oxygen supply is dependent mainly on coronary blood supply. The coronary artery diameter declines passively when the lumen is narrowed by intimal hyperplasia or atherosclerosis, or by the formation of a secondary thrombus in a susceptible coronary artery segment. In addition to that, the mechanisms regulating coronary artery diameter may be altered in such a way that contraction of the coronary artery smooth muscle contributes actively to the already present luminal narrowing. In a small group of patients, vasoconstriction may be solely responsible for myocardial ischemia. The coronary artery mechanisms underlying disturbed coronary artery flow regulation are outlined in chapter 2.

For now it must be emphasized that the present chapter on clinical aspects of ischemic heart disease is bound to be incomplete when considering the voluminous detailed text books on this topic. However, this chapter should be read as a prologue, setting the stage for hypotheses and experimental investigations, which are only a modest reflection of clinical reality described here.

1.2 Categories of ischemic heart disease

Ischemic heart disease is comprised of a spectrum of related syndromes, with related symptoms and pathological mechanisms. The spectrum reaches from silent ischemia on one end to acute transmural infarction and sudden cardiac death on the other end.

In *stable angina pectoris* the most important symptom is the typical anginal discomfort in the chest, which usually begins gradually and reaches a maximal intensity over a period of minutes, before disappearing within 20 minutes after cessation of the precipitating activity, or within less than approx. 10 minutes, when nitrates are administered. The symptoms should be present over a longer period of time (>60 days), and remain constant. Anginal pain on the chest was described a long time ago:

> "... But there is a disorder of the breast marked with strong and peculiar symptoms, considerable for the kind of danger belonging to it, and not extremely rare, which deserves to be mentioned more at length. The seat of it, and the sense of strangling and anxiety with which it is attended, may make it not improperly be called angina pectoris ..."
>
> William Heberden, 1772 [5]

Nowadays we would describe typical angina pectoris as a discomfort in the chest or adjacent areas, caused by myocardial ischemia and associated with a disturbance of myocardial function, but without myocardial necrosis. Obviously, it is important to differentiate angina pectoris from other, non-ischemic, non-cardiogenic causes of chest pain. These include oesophageal reflux or spasm, acute pancreatitis, pulmonary hypertension, and many others. Although the ECG is not necessarily abnormal, abnormalities may include changes of the ST-segment, in particular during provocative exercise testing. Stable angina pectoris is induced by an increased oxygen demand, like in physical activity (as in provocative exercise testing), hurrying, eating, emotions, but also in situations of excessive metabolic demand like in fever, thyrotoxicosis, severe anaemia, and hypoglycaemia. In stable angina an obstruction of the blood vessel results in a decreased but fixed coronary flow reserve. Characteristically, the patient can predict with some precision which level of activity will cause angina. This has also been called 'fixed threshold angina' [6, 7]. However, many patients have 'good days', when patients are able to perform substantial physical activity, or 'bad days', when even small physical activity can cause symptoms of myocardial ischemia. The latter form of angina has also been called 'variable threshold angina'. A number of patients suffer from episodes of myocardial ischemia, which are not always accompanied by the characteristic angina

pectoris. During this 'symptom-free' ischemia, changes of the ECG and a reduced myocardial perfusion may be observed [8]. This type of ischemia is denoted as *silent ischemia.* Silent ischemia may be present in patients who do not perform exercise intensely enough to provoke the symptomatic ischemia, in patients with diabetes mellitus, and in patients in which the pain threshhold is higher than in others [9]. It may also occur in non-symptomatic intervals in patients previously characterized as stable or unstable angina pectoris, or in patients with a previous myocardial infarction.

Unstable angina pectoris is a clinical syndrome which requires particular interest because of its intense and frightening nature, and because of the fact that many patients with acute myocardial infarction reported symptoms resembling unstable angina, prior to infarction [10]. The population of patients with unstable angina is characterized by one or more of the following signs: (*i*) crescendo angina, i.e. more severe, frequent, or prolonged attacks, superimposed on an existing pattern of stable angina; (*ii*) angina at rest as well as in minimal exercise; (*iii*) angina of recent onset (< 60 days), brought on by minimal exercise. In general, responsiveness to nitrates is less than in stable angina. Changes of the ECG are often seen, but disappear with cessation of the pain. The specific enzymatic changes and persistent ECG alterations as in acute myocardial infarction should be absent. Many investigators also include a poor response to nitroglycerin or rest. The pathophysiology of unstable angina is more complex and dynamic than in stable angina. In unstable angina, progression of atherosclerotic lesions, (resulting in) altered vasomotor tone and endothelial damage, the rupture of existing plaques and platelet aggregation and thrombosis, may all together, or separately, contribute to the disease [11]. Due to the obvious patient heterogeneity in nature and severity, and the important differences in already applied therapeutic measures, a classification has been developed to differentiate between subgroups [12].

In contrast to stable and unstable angina, *myocardial infarction* is characterized by irreversible myocardial damage or necrosis, followed by chronic functional changes and scarring of the involved myocardium. Myocardial infarction is caused by atheromatous narrowing of the arterial lumen, often in combination with the rupture of an existing atherosclerotic plaque, a superimposed occluding thrombus, and/or vasospasm [13, 14]. Where the myocardium is damaged to a severe extent, the patient may develop cardiogenic shock, with a cyanotic appearance of cool, but clammy skin. Activation of vagal reflexes results in nausea and vomiting in a majority of patients (in particular in inferior myocardial infarction), and feelings of weakness and dizziness are common. The chest pain is variable, but may be very severe, and lasts for more than 30 minutes to a number of

hours, and does not usually respond to nitrates (the latter two in contrast to stable and unstable angina pectoris). In 20-60% of non-fatal myocardial infarctions, the infarction remains unrecognized or is not perceived at all. These cases, which are recognized in routine ECG or post-mortem investigations, are called silent myocardial infarction [15].

Since the discovery of elevated levels of serum glutamic oxaloacetic transaminase (SGOT) in patients with myocardial infarction [16], the levels of SGOT and other cardiac enzymes in serum have become an important tool in the diagnosis of irreversible myocardial cell injury. At present the most widely applied enzyme test is the detection of creatine kinase (CK), which exceeds the normal serum concentrations within 4-8 hours after onset of myocardial infarction and returns to normal within 3-4 days after the onset of chest pain. Three iso-forms of CK exist (MM, MB, and BB) of which CK-MB is the most specific for damage of cardiac myocytes [17, 18]. Other enzyme tests include lactate dehydrogenase (LDH). This enzyme consists in five isoenzyme forms, of which LDH_1 is the most specific for the cardiac myocytes [19]. Apart from total LDH activity, the ratio of LDH_1 / LDH_2 may also be measured, in which ratios of over 0.76 [19] or 1.0 [20] point at a previously experienced infarct. This enzyme exceeds normal levels by 24-48 hours, reaches a maximum in 3-6 days, and is back to normal levels in 8-14 days. Therefore, LDH isoenzyme analysis should be reserved for cases in which creatinine kinase levels have returned to normal levels (i.e. after 3-4 days). Measurement of aspartate aminotransferase (AST: similar to the above mentioned SGOT test) is less specific and does not add to information obtained by measuring activity of CK-MB or LDH [21].

Variant angina pectoris was described in 1959 by Myron Prinzmetal and co-workers [22] as a distinct syndrome, characterized by angina pectoris at rest (therefore also: *Prinzmetal's angina*). Variant angina may lead to myocardial infarction, cardiac arrhythmias, and sudden death, but 5 year survival is relatively good (around 90%) [23]. In contrast to (un)stable angina, variant angina is not provoked by exercise. Moreover, angina at rest in variant angina patients has usually not progressed from an earlier stage in which angina would be provoked by decreasing amounts of exercise, like in many patients with unstable angina. During the attack, typical ST-segment alterations may occur in the ECG. Coronary arteries of patients with Prinzmetal's angina constrict with abnormal sensitivity to ergonovine infusion [24]. Although the coronary angiogram of some patients reveals severe coronary obstructions, these are not necessarily present.

1.3 Epidemiology

Cardiovascular diseases are the most common cause of death in the western world. In the Netherlands in 1992, 40% of the total death count was due to cardiovascular disorders (see Ref. 25). 40% of the people dying of cardiovascular disease, died of ischemic heart disease, whereas 24% died of cerebrovascular accidents. Among those dying of ischemic heart disease, the major proportion of individuals suffered from acute myocardial infarction (77%). Thus, acute myocardial infarction is the most common single cause of death in the Netherlands with a total number of 16.248 recorded fatalities in 1992.

Males are more at risk of dying of ischemic heart disease than females, and the risk increases with age. The risk for females of dying of acute myocardial infarction is similar to that of males who are ten years older.

Since 1972, the age-related death rate due to ischemic heart disease has shown an impressive decrease of 43% and 44% for males and females, respectively (age-standardized for 1992, per 100.000). In contrast, the standardized hospital admission rate showed a constant increase since 1972. The combination of a decreased death rate and an increase in hospital admissions may point at improved survival rates or at an increase of the number of admissions for diagnostic or interventional purposes (De Nederlandse Hartstichting, 1994 [25]).

Public awareness of risk factors is considered a substantial contribution to the decline in death rate to ischemic heart disease. The three main risk factors in ischemic heart disease are dyslipidaemia, smoking, and hypertension. Risk factors often co-exist, and may act synergistic, for example in smoking-related hypertension.

Dyslipidaemia is a complex of disorders, referring to abnormal metabolism of plasmalipids, which can be caused by genetic factors and by diet, or can be secondary to other diseases like diabetes. In particular the ratio of low density lipoproteins (LDL) compared to high density lipoproteins (HDL), and also the serum cholesterol concentrations are now well established risk factors. It was shown, for instance, that 45% of the coronary artery disease differences between the male populations of different countries, could be accounted for by the variation in serum cholesterol levels. Variability in HDL levels accounted for 32% of differences, whereas the ratio of total serum cholesterol to HDL accounted for 55% of mortality differences [26]. Moreover, a number of studies reducing total serum cholesterol by pharmacological or dietary means have shown significant reductions in mortality due to ischemic heart disease compared to placebo

treatment [27, 28]. Such measures have also been shown to reduce development of atherosclerosis [29], and may even cause regression of existent atherosclerotic lesions [30, 31].

Smoking of cigarettes is another major risk factor in ischemic heart disease. Several mechanisms of action have been proposed, for example a negative effect on HDL/LDL ratio's [32], or increased platelet aggregation [33]. Furthermore, nicotine may stimulate the sympathetic nervous system, leading to the release of noradrenaline, which may result in increased coronary tone [34]. Cessation of smoking could reduce the risk of myocardial infarction within 2 years [35, 36], but others have shown that even men, who have given up smoking more than 20 years ago, have an increased risk of myocardial infarction [37].

Also hypertension was shown to predispose for coronary atherosclerosis [38]. Although a beneficial effect of lowering blood pressure on the incidence of stroke was reported, the impact on ischemic heart disease (if any) was much less pronounced [39]. Other risk factors include lack of physical activity, a family history of ischemic heart disease, diabetes mellitus, obesity, and perhaps mental stress or personality.

1.4 The current therapeutic approach of ischemic heart disease

The therapy of ischemic heart disease is primarily aimed at minimizing anginal pain and possible consequential damage of the myocardium. This can be achieved by increasing the supply of oxygen through augmentation of coronary flow, or by decreasing the demand of oxygen via suppression of myocardial contractility or heart rate. Secondly, therapy is aimed at prevention of further myocardial ischemic events. In myocardial infarction, the therapeutic approach is also aimed at support and restoration of myocardial function when necessary.

Like in other syndromes of ischemic heart disease, the treatment of *stable angina pectoris* should first be aimed at the elimination of predisposing risk factors, such as hypertension and smoking. The treatment of obesity is also important in that an ideal body weight raises the threshhold of angina pectoris. A similar effect may be achieved by treating anaemia, fever, or pulmonary disease, which all have a detrimental effect on the oxygen supply/demand balance. Pharmacological treatment consists of nitrates, beta-adrenoceptor antagonists, and calcium channel blockade [40]. Nitrates act by inducing vasodilatation in both venous and arterial blood vessels. Dilatation in venous blood vessels helps to reduce the preload, which relieves wall stress in the ventricles [41]. In arteries,

vasodilatation alleviates ventricular afterload, and small changes in coronary artery diameter contribute to a decreased coronary artery flow resistance [42]. Side effects include headache, flushing and hypotension. Antagonists at beta-adrenoceptors act by attenuating the positive inotropic and positive chrontropic responses to catecholamines on the myocardium (mediated primarily via $beta_1$ receptors). Since sympathetic catecholaminergic stimulation takes place during activity and excitement, this class of drugs is particularly effective in increasing the threshhold of angina pectoris [43]. Because stimulation of $beta_2$ receptors induces bronchodilatation, and because even so-called cardioselective ($beta_1$) receptor blocking agents have only relative selectivity for $beta_1$ receptors, beta-adrenoceptor antagonists should better not be prescribed to susceptible individuals, like asthma patients. Since $beta_2$ receptors may also mediate dilatation of peripheral blood vessels, intermittent claudication is considered a relative contraindication for treatment with beta receptor antagonists. Calcium antagonists act by decreasing the entry of calcium into smooth muscle cells and cardiomyocytes, thereby interfering with contraction, leading to vasodilatation and a negative inotropic response [44]. Recent data suggest that calcium antagonists may also help to attenuate the development of new atheromatous lesions [45, 46]. Because the mode of action and the haemodynamic profile of nitrates, beta adrenoceptor antagonists, and calcium antagonists, is clearly different, several combination regiments have been suggested for patient subgroups of stable angina and have been established to be beneficial in large clinical trials.

Non-pharmacological interventions include coronary artery bypass surgery, percutaneous transluminal coronary angioplasty (PTCA) sometimes combined with intra-arterial stent implantation, or laser angioplasty, or directional transluminal coronary atherectomy. 'Significant disability from moderate to severe angina pectoris, unresponsive to optimal medical care' remains a valid indication for the above mentioned interventional methods of treatment, although exact indications develop continuously as expertise increases [47, 48]. Patients with depressed left ventricular function have better 10 year survival when treated with bypass surgery than after medical therapy, but medically-treated patients with mild stable angina and normal left ventricular function have a survival similar to patients assigned to surgery [49]. Depressed left ventricular function below a critical level (ejection fraction <20%) is associated with increased operation mortality [50]. Quality of life is increased in patients undergoing surgery during the first ten years, but the difference disappears thereafter [51]. The use of internal mammary artery bypass grafts in bypass surgery, has considerable advantages over the use of saphenous vein in 10 year survival [52], but occlusion of the graft is still a common problem of coronary artery bypass surgery. The overall chance of occlusion at 10 years after surgery is 40-50% per distal anastomosis.

Occlusion may occur at an early stage (before hospital discharge, due to e.g. technical difficulties, like kinks) or over many years, due to neointimal formation and occlusion with an atherosclerotic plaque [53]. Treatment with aspirin (with or without dipyridamole [54, 55]), and lowering of the LDL cholesterol / HDL cholesterol balance [56, 30], have been shown to be associated with prolonged patency of bypass grafts.

The 'ideal lesion' for treatment with PTCA consists of a single, smooth, concentric lesion in an easily accessible proximal coronary artery segment [57]. For patients with single-vessel coronary artery disease, PTCA offers earlier and more complete relief of angina than medical therapy [58]. Coronary arteries with excessive tortuosity, or near complete obstruction are less suitable for PTCA [59]. The main and still unresolved problem is the frequent occurrence of chronic restenosis following PTCA. Between 20 and 30% of patients treated with PTCA, develop renarrowing of the dilated segment (restenosis), and subsequent recurrent myocardial ischemia within 6 months after treatment. Platelet-derived vasocontrictors (e.g. 5-HT, thromboxane A_2) and/or mitogens (e.g. platelet-derived growth factor or PDGF), released after platelet adherence to the luminal surface of the blood vessel after dilatation, are believed to be involved in this process [60]. Also other vasoconstrictors/mitogens, like angiotensin II, and endothelins have been implicated in the process of restenosis. However, medical treatment with aspirin [61], 5-HT receptor antagonists [62], thromboxane receptor antagonists [63], and angiotensin converting enzyme inhibitors [64], have not been successful in the prevention of restenosis, despite promising previous results in animal studies [65]. By contrast, the use of balloon expendable coronary stent implantation has recently been shown to reduce the rate of restenosis, when compared to balloon angioplasty [66], and treatment with antibody fragments aimed at platelet glycoprotein IIb/IIIa integrin (mediating the final common pathway leading to platelet aggregation), resulted in reduction of the need for subsequent coronary revascularization procedures in the first six months [67].

Treatment of *unstable angina pectoris* is relatively similar to treatment of patients with stable angina, considering the use of nitrates, beta blocking drugs, and calcium antagonists, and non-pharmacological interventions. However, in unstable angina the use of anticoagulants and antiplatelet drugs is often recommended, which reflects the possible involvement of platelet activation and thrombus formation. Indeed, both aspirin and heparin, and their combination have been shown to improve clinical outcome of patients with unstable angina [68, 69]. Also PTCA and surgical coronary artery bypass grafts result in immediate abolition of ischemic episodes [70, 71]. The effectiveness of thrombolysis (vide infra) in unstable angina is still uncertain [72, 73] and may be detrimental [74].

The treatment of *myocardial infarction* is essentially different from the treatment of other ischemic heart disorders, due to the acute and near complete occlusion of the artery, and due to the resulting irreversible damage of the myocardium. After diagnosis and hospitalization, general measures of treatment include oxygen, bedrest, and analgesics. Immediate measures should furthermore be aimed at the reperfusion of the ischemic myocardium [75]. This can be achieved by thrombolysis using streptokinase or recombinant tissue-type plasminogen activator (rt-PA), which are both very effective in the first hours after the infarction [76, 77]. Aspirin, added to streptokinase, results in further improvement of vascular mortality rate [78]. Patients in which reperfusion cannot be achieved using thrombolysis, may benefit from rescue PTCA [79], and sometimes emergency bypass surgery [80]. Others even claim a small beneficial result of immediate PTCA compared to thrombolysis [81, 82]. Beta-adrenoceptor blockade early after infarction resulted in significant reduction of mortality [83], but the use is contraindicated when common complications of myocardial infarction, like heart failure and heart block, are present. Intravenous administration of nitrates limits infarct size and the rate of complications, and improves post-infarction remodelling [84]. Calcium antagonists, however, have not been shown to be effective in reducing mortality and infarct size [85]. Relatively new therapeutical approaches, currently under investigation to achieve early reperfusion include additional thrombin inhibitors [86]. Other drugs used in acute myocardial infarction are aimed primarily at the prevention of arrhythmias, hypotension, heart-failure, and shock, and at a beneficial effect on postinfarction remodelling. These drugs are not within the scope of this thesis.

In contrast to patients with myocardial infarction, patients with *variant or Prinzmetal's angina* respond extremely well to calcium antagonists but not to beta receptor antago-nists [87]. Nitrates are usually effective to abolish the attack [88]. PTCA is only rarely indicated in patients with Prinzmetal angina, when additional obstructive lesions are present [89].

1.5 Myocardial ischemia induced by anti-migraine drugs

Anti-migraine drugs, like ergotamine and sumatriptan, may induce myocardial ischemia in some patients. Especially ergotamine, in use since many decades is renowned for its myocardial side effects. Therapeutic doses of ergotamine were shown to be associated with myocardial ischemia, myocardial infarction, cardiac arrest, and sudden

death [90]. The development of the novel anti-migraine drug sumatriptan was aimed at the development of a drug that would selectively stimulate the 5-HT_1 receptor in dilated extracerebral blood vessels. However, also this novel drug was found to lead to symptoms of angina in some patients [91, 92]. In exceptional cases, changes of the ECG have been recorded [93]. However, ECG abnormalities are usually absent in patients presenting with symptoms resembling angina, after taking anti-migraine drugs (W.H. Visser, personal communication). Patients suffering from cardiac side-effects do not necessarily belong to high risk categories for ischemic heart disease [91]. Therefore, it has been speculated that some patients may be hyperresponsive to stimulation of their contractile coronary artery 5-HT_1 receptors. The mechanisms involved in this putative hyperresponsiveness are currently under investigation. An alternative explanation for the chest-symptoms may be an effect on lungs or the oesophagus [94]. Although nitrates are effective in the treatment of anginal symptoms after anti-migraine drugs, symptoms usually disappear spontaneously. Lastly, it must be kept in mind that alternative anti-migraine drugs, like ergotamine and dihydroergotamine, are clearly more potent vasoconstrictors of the human isolated coronary artery [95, 96], and therefore appear more likely to induce cardiac ischemia.

1.6 Conclusions

In summary, ischemic heart disease is a spectrum of related disorders in which the oxygen supply does not meet the demand. This may result in stable, unstable or variant angina, or in myocardial infarction. All are usually due to both passive obstruction by thrombus plugging or plaque formation, and also to active vasoconstriction caused by vasoconstrictor agonists. The contribution of active vasoconstrictor obstruction may vary from very little in case of fixed obstruction in stable angina, upto almost hundred percent in case of variant angina pectoris. Treatment is aimed at reducing myocardial ischemia, and related anginal pain, and at early reperfusion and prevention of haemodynamic complications in myocardial infarction. Large scale clinical trials are being undertaken to establish the best choice of treatment for different groups of patients.

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Chapter 2

Physiology and pathophysiology of vasomotor tone

2.1 Introduction

The supply of oxygen to an organ is largely dependent on blood flow. Blood flow is determined by the pressure gradient and by resistance along the blood vessel. When blood flow in an artery is considered as flow of fluid through a cylinder, resistance is determined by the length of the cylinder, by viscosity of the fluid, and most importantly, by diameter of the cylinder. Diameter of the blood vessel is dependent on its anatomical constitution (i.e. the natural diameter, and the presence or absence of luminal obstruction) and by vasomotor tone. This chapter describes mechanisms underlying vasomotor tone. Vasomotor tone is defined as the outcome of contractile and relaxant forces within the blood vessel wall. Although contraction and relaxation both result directly from processes in the vascular smooth muscle, it has now become evident that the endothelial lining is an important relaxant and contractile determinant of smooth muscle tone. Vasomotor tone is governed locally, via *in situ* release of contractile and relaxant mediators, and systemically, via hormones released elsewhere. Disturbance of the equilibrium between relaxation and contraction is believed to be an important determinant of ischemic disease.

2.2 Smooth muscle physiology

Structure of the contractile apparatus

Interaction between the major contractile proteins actin and myosin generates vascular smooth muscle tone. Heads of the bundled myosin thick filaments bind to actin thin filaments in an, ATP driven, cyclic fashion, thereby generating tension as a result of conformational changes in the myosin head when bound to the actin filament. During this cycle, the angle of the myosin head is translocated from 90° to 45° with respect to the longitudinal axis of the actin-, and myosin-containing filaments [1, 2]. The structure of the smooth muscle cell contractile apparatus is ordered in a less apparent

manner than the structure of striated muscle, in which light microscopy may suffice to observe the organization of actin and myosin filaments. In smooth muscle, thin filaments containing actin and tropomyosin are attached to the cell membrane and to dense bodies which contain, among other proteins, α-actinin [3]. Dense bodies may be compared to Z-lines of striated muscle [4]. Additional three-dimensional networks, consisting of the proteins desmin and vimentin interconnect dense bodies, resulting in an extensive cellular skeleton [5].

Interaction of actin and myosin

Vascular myosin consists of two heavy chain subunits (MW 204.000 Da and 200.000 Da) and of two sets of light chain subunits: regulatory 20.000 Da (hence LC20) chains and alkali 17.000 Da (hence LC17) chains. Myosin light-chain kinase (MLCK) phosphorylates the serine-19 of the LC20. MLCK itself is activated by Ca^{2+}, and by the calcium binding protein calmodulin [6]. Phosphorylation of the LC20 initiates the cycle of actin-myosin cross-bridging, and thus initiates contraction. In particular, the phosphorylation of the LC20 leads to activation of myosin Mg ATPase activity, which also requires the presence of actin (therefore also 'actin activated myosin Mg ATPase') [7]. The subsequent ATP hydrolysis provides the energy required for cycling of cross bridges. Cycling of cross bridges refers to the cycle of attachment of the actin binding domain of the globular head of myosin to the actin filament, the tilting of the myosin head, leading to translocation accompanied by the release of ADP and P_i, the detachment of the actin chain, the re-tilting, and the subsequent re-attachment further down the actin filament. The state of phosphorylation of the myosin light chain is reversed by dephosphorylation by protein phosphatase (MLCP, myosin light chain phosphatase) and by modulation of MLCK activity. Indeed, MLCK can be phosphorylated by several protein kinases, like cAMP- and cGMP-dependent protein kinase [8], and by protein kinase C [9] (for schematic representation, see Figure 1). Other actin-binding proteins that modulate contractility have been identified more recently. First, it has been suggested that calponin modulates Ca^{2+} sensitivity of smooth muscle contraction [1], or the effects of protein kinase C on smooth muscle contraction [10]. Secondly, phosphorylated caldesmon has been suggested to slow actin-myosin crossbridge detachment [11, 12]. However, little is known in detail about the latter two presumed modulators of contraction.

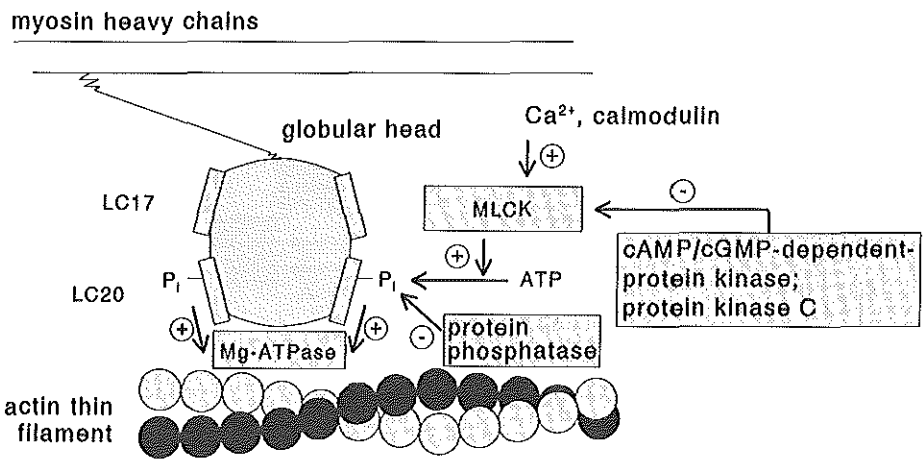

Figure 1. Schematic representation of interaction of myosin and actin. +, positive effect/induction; -, negative effect/inhibition; MLCK, myosin light-chain kinase; LC17, myosin light chain, 17.000 Da; LC20, myosin light chain, 20.000 Da; Mg-ATPase, myosin Mg^{2+} ATPase.

Latch state

In intact vascular smooth muscle, the extent of LC20 phosphorylation correlates best with the velocity of muscle shortening at zero-load, and is an estimate of the rate of actomyosin crossbridge cycling. Vascular smooth muscle contraction induced by electrical or pharmacological stimulation is characterized by rapid increases of LC20 phosphorylation and velocity of muscle shortening, followed by a gradual return to near-basal levels of both shortening velocity and LC20 phosphorylation. Tension, however, increases more slowly, but is maintained while the LC20 phosphorylation level decreases [13]. In this state of 'tension maintenance', cross bridges cycle at about one-quarter of the normal cycling rate, which allows the smooth muscle to maintain contraction at low energy cost. This state has been called 'latch state' by Hai and Murphy [14], who hypothesized that dephosphorylation of the LC20, while in high affinity binding conformation, would alter the kinetics of crossbridge detachment, thereby prolonging actin-myosin binding, resulting in sustained tension.

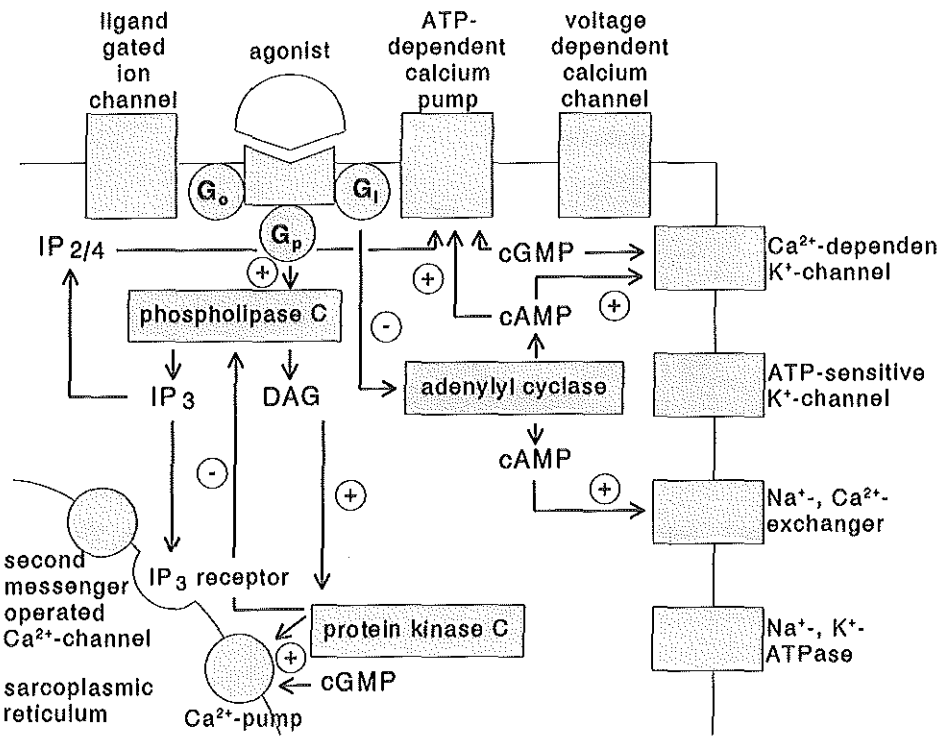

Figure 2. Schematic representation of signal transduction and excitation contraction coupling in the smooth muscle cell. +, positive effect/induction; -, negative effect/inhibition; DAG, diacylglycerol; IP_3, Inositol-1,4,5-triphosphate.

Excitation-contraction coupling

Cytoplasmic calcium is a primary determinant of tension generation. Calcium is present in the extracelluar compartment in millimolar concentrations, whereas the cytoplasmic resting concentration is around 100 nM. The pathways leading to a contractile response require only up to approximately 600-800 nM Ca^{2+} [15], for which the sarcoplasmic reticulum is an important source. Several mechanisms affect the concentration of calcium in the cytoplasm (for schematic representation, see Figure 2).

The cellular membrane is equipped with channels that, when activated, allow for a rapid influx of Ca^{2+} into the cell. These calcium channels may be opened by depolari-

zation of the membrane potential (voltage-dependent calcium channels, see Ref. 16). Membrane potential is determined by the concentration of the main anions and cations across the membrane. The concentration of ions is dependent on various ion transporting systems in the membrane, like Ca^{2+}-dependent and ATP-sensitive potassium channels (K^+_{Ca} and K^+_{ATP}) [17-19], Na^+-K^+ ATPase [20], and a Na^+-Ca^{2+} exchanger. The latter utilizes the electrochemical Na^+ gradient across the cell membrane for concomitant cellular Ca^{2+} extrusion [21]. When the K^+ channels are activated, and the K^+ conductance increases, hyperpolarization follows, leading to inactivation of voltage dependent Ca^{2+} channels and enhanced Ca^{2+} extrusion via the mentioned Na^+-Ca^{2+} exchanger. Relaxation, or reduction of vasomotor tone follows. K^+_{Ca} channels may be activated by 5'-GMP, the major metabolite of cGMP, which is formed after stimulation with e.g. nitrovasodilators and endothelium-derived relaxing factor (EDRF) [22]. K^+_{ATP} channels can be activated by pharmacological agents like minoxidil and various neuropeptides, and can be inhibited by compounds like glibenclamide [23].

To remove Ca^{2+} from the cytoplasm, the cell utilizes outwardly directed ATP-dependent Ca^{2+} pumps [24] as well as the above mentioned Na^+-Ca^{2+} exchanger [21]. It has been shown that these Ca^{2+} pumps are stimulated by compounds that increase cGMP like nitrovasodilators, in which the generation of phosphatidylinositol diphosphate appears to be involved as well [25].

The membrane of the sarcoplasmic reticulum contains Ca^{2+} channels which are activated by the second messenger inositol triphosphate and are therefore referred to as second messenger operated calcium channels [6] (vide infra). In addition, the sarcoplasmic membrane contains a Ca^{2+} pump, which actively stores Ca^{2+} in the sarcoplasmic reticulum [27]. It has been suggested that the affinity of this sarcoplasmic reticulum Ca^{2+} pump is enhanced by agents that increase cGMP, whereas protein kinase C is believed to increase the maximal activity of the pump [28].

Signal transduction

Binding of an agonist to its receptor induces a cascade of biochemical events, ultimately leading to relaxation or contraction. α- and ß-adrenergic, muscarinic, 5-HT-, angiotensin, arginine vasopressin, and endothelin receptors are all coupled to a specific intracellular second messenger system via guanine nucleotide (GTP, GDP) binding (G) proteins. These proteins consist of three subunits, α, ß, and γ. Receptor activation can induce the replacement of GDP by GTP in the α subunit, thereby altering the conformation of the protein and making the active site of the G-protein available for

activation or inhibition of enzymes or ion channels. Different G proteins exist in vascular smooth muscle. G_s protein stimulates adenylyl cyclase, whereas G_i protein inhibits adenylyl cyclase. G_p activates phospholipase-C, and G_o proteins activate ion-channels (reviews, see Ref. 29 and 30). Receptors, mediating contraction after stimulation with 5-HT are, for instance, coupled to G_i- (5-HT_1) and G_p (5-HT_2) proteins [31]. Endothelin receptors and vasopressin V_1 receptors are usually believed to be coupled to G_p proteins [32, 33].

Stimulation of phospholipase-C induces the conversion of the membrane constituent phosphatidyl-inositol 4,5-biphosphate (PIP_2) to yield inositol triphosphate (IP_3) and diacylglycerol (DAG) (see Ref. 34). IP_3 binds to receptors on the membrane of the sarcoplasmic reticulum, resulting in release of Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm [26]. Diacylglycerol activates protein kinase C. Protein kinase C may be stimulated experimentally by phorbol esters [35], which interact with the DAG binding site. Protein kinase C inhibits the activation of phospholipase C [36], and modulates the phosphorylation of various ion channels and -exchangers [37-39].

Adenylyl cyclase catalyses the formation of adenosine-3',5'-monophospate (cAMP) from adenosine triphosphate (ATP). cAMP may induce activation of K^+ channels, resulting in hyperpolarization, decreased Ca^{2+} influx, and relaxation [16]. Increased levels of cAMP also lead to enhanced Ca^{2+} cytoplasmic extrusion [40], which again results in relaxation. Finally, cAMP-induced activation of cAMP-dependent protein kinases leads to the phosphorylation of myosin light chain kinase (MLCK), thereby decreasing the Ca^{2+}-sensitivity of the contractile apparatus, resulting in relaxation [41]. Also the accumulation of cyclic guanosine monophosphate (cGMP) following stimulation of guanylate cyclase will result in relaxation via comparable mechanisms [42, 43].

2.3 Physiology of the endothelium

Endothelium-dependent relaxation

In 1980 Robert Furchgott and John Zawadzki [44] described that the response of rabbit isolated aorta is dependent on the presence of endothelium. Endothelium-intact aorta preparations relaxed after exposure to acetylcholine, whereas rings, in which the endothelium had been rubbed, did not or even responded with contraction. It was

shown that the endothelium produced a very labile, relaxing substance, which was not a prostanoid. It was decided that the endothelial factor involved would be referred to as endothelium-derived relaxing factor (EDRF [45]). Co-workers of Robert Furchgott decided that endothelium-derived relaxing 'factor' was to be preferred over 'substance' or 'material', basically because the abbreviation EDRF would contain its inventor's initials [46].

Although several hypotheses had been proposed, the chemical identity of EDRF remained unknown. Even before the identification of the factor involved, it had been shown that EDRF would induce guanylate cyclase, thereby increasing levels of cGMP, ultimately resulting in vasodilatation [47]. It was not until 1987 that EDRF was proposed to be similar to nitric oxide (NO) [48, 49]. Palmer and co-workers also showed that nitric oxide was synthesized from L-arginine, resulting in the production of nitric-oxide and citrulline [50]. False precursors of nitric oxide, like N^G-monomethyl-L-arginine, inhibited the synthesis of nitric oxide [51, 52]. By use of these 'NO-synthase inhibitors' a basal dilatory nitric oxide tone could be shown [53, 54], which contributed continuously to vasomotor tone. Moreover, it was shown that a large number of endogenous mediators, including those released by aggregating platelets [55], could induce the endothelial production of EDRF [56, 57]. Apart from platelet-induced, EDRF-mediated relaxation of the vascular smooth muscle, nitric oxide also attenuated the aggregation and adhesion of platelets [58, 59], thus offering two-way protection against platelet-induced vasoconstriction.

Prostacyclin

Also prostacyclin [60, 61] is released by the vascular endothelium in response to a number of stimuli. Endothelial cells produce 10-20 times more prostacyclin than smooth muscle cells [62], but its production is not as confined to the endothelium, like in case of nitric oxide. Strictly speaking, prostacyclin is therefore not entirely an *endothelium*-derived relaxing factor. Prostacyclin is released by the endothelium in response to shear stress, hypoxia, and receptor-operated mechanisms. Receptor operated mechanisms include the release of prostacyclin in response to activation of receptors for acetylcholine, thrombin, and histamine (review, see Ref. 63). Prostacyclin may also be released under basal conditions and contributes to unstimulated vasomotor tone, and may induce relaxation via a cAMP-dependent mechanism [64]. Prostacyclin may be released in response to platelet products like platelet-derived growth factor [65], and inhibits both platelet adhesion and activation [61]. Thus, prostacyclin is a physiological

antagonist of thromboxane A_2, both as an inhibitor of platelet aggregation and as a vasodilator.

Endothelium-derived contracting factor

Under certain conditions the endothelium may release mediators that induce or augment contraction of the vascular smooth muscle. This suggestion was based on experiments in canine femoral arteries, in which removal of the endothelium reduced contractions evoked by potassium [66]. Several endothelium-derived mediators have been shown to modulate contractile responses of the underlying smooth muscle.

First, it has been postulated that metabolites of arachidonic acid are involved in endothelium-dependent contractions. Indeed, arachidonic acid may induce contractions of canine veins, dependent on the presence of endothelium. These responses may be attenuated by inhibitors of cyclooxygenase [67]. Secondly, it has been shown that anoxic conditions lead to augmented contractions to a number of stimuli. In a 'sandwich bioassay preparation' it was shown that the hypoxic endothelium dependent contractile factor may be delivered from an endothelium-intact donor-vessel to a coronary artery strip without endothelium [68, 69]. The exact nature of this hypoxic endothelium dependent contractile factor remains unknown. Thirdly, it has been shown that a 21 amino acid peptide is released upon endothelial stimulation using a variety of endogenous mediators, like thrombin, vasopressin, and angiotensin II [70]. This peptide is endothelin (ET) [71], of which three endogenous isopeptides have been identified (ET-1, ET-2, and ET-3). Recent evidence suggests that endothelin plays a continuous role in the maintenance of blood pressure, not only as a tonic substance [72], but possibly also as a dilator compound [73].

2.4 Synopsis: disturbed vasomotor tone

The above has shown that vasodilatation and vasoconstriction both result from mechanisms within the vascular smooth muscle and the endothelium. These dilatory and contractile forces result in a balance, which, when disturbed, may be involved in various disease states. For instance, an increased endothelium-dependent dilatory response appears connected to endotoxin-induced septic shock [74], whereas a dysfunctional endothelium may result in increased vasoconstrictor responses of the coronary arteries [75, 76]. Moreover, diseased endothelium may result in increased aggre-

gation of platelets at subendothelial collagen, and enhanced exposure of underlying smooth muscle to vasoconstrictor compounds. Thus, a disturbed balance may contribute to a variety of cardiovascular disorders, including cardiac ischemia.

Alteration of the contraction-relaxation equilibrium due to atherosclerosis

It has now been established that atherosclerosis results in alteration of function of the endothelium. This endothelial disorder may be due to a reduction in the release of EDRF from endothelial cells. This was observed in functional studies measuring relaxation in human coronary arteries *in vitro* [77], and *in vivo* [78]. In the human isolated coronary artery, both stimulated and basal release of nitric oxide were reduced [79]. Shimokawa and co-workers [80] suggested that atherosclerosis-related impairment of endothelium-dependent relaxation is due to specific damage of a pertussis-toxin-sensitive endothelial G_i protein, connected to endothelial 5-HT_1- and α_2-receptors. In human coronary arteries, the small relaxant response to stimulation of endothelial muscarinic receptors was entirely abolished in atherosclerotic blood vessels, whereas the response to substance P, or calcium ionophore A23187, remained relatively intact [81]. This would suggest that atherosclerosis affects specific aspects of endothelium-dependent relaxation. Lastly, evidence has been provided that EDRF impairs the release of the endothelium-derived contracting factor, endothelin [82]. Hence, in atherosclerotic vessels with impaired EDRF release, the production of endothelin may well become enhanced.

Whether formation of neointimal hyperplasia leads to an increased barrier of diffusion is yet uncertain. In the rabbit carotid artery, formation of intimal hyperplasia did not reduce endothelium-dependent relaxation [83]. Alternatively, it has been hypothesized that reduced smooth muscle sensitivity to EDRF, may be involved in the reduced responses to endothelium-dependent vasodilators. Vasodilatation by nitrovasodilators remained intact in perfused atherosclerotic rabbit carotid arteries [84], and vasodilatation in atherosclerotic human isolated coronary arteries was decreased only in case of low concentrations of glyceryl trinitrate [81]. However, others have observed a clearly decreased sensitivity to the nitrovasodilators SIN-1 and nitroglycerin, suggesting that the nitrovasodilator susceptibility of guanylate cyclase may be altered in atherosclerosis [85].

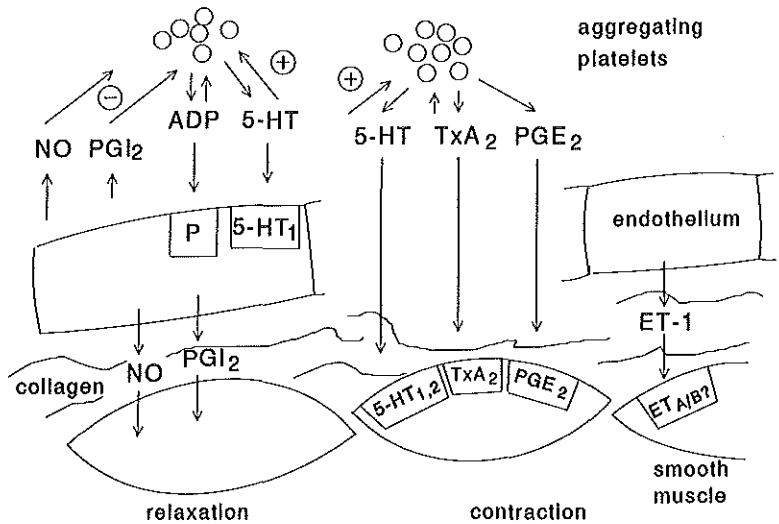

Figure 3. The balance between mediators of vasoralaxation and mediators of vasoconstriction. Mediators may be released by the endothelium or, for instance, by platelets which are activated upon exposure to subendothelial collagen. +, pro-aggregatory effect; -, anti-aggregatory effect.

There is still considerable debate whether alteration of responses is due solely to endothelial damage or whether hypercontractility of vascular smooth muscle could also contribute to the altered equilibrium. Fukai and colleagues [86] demonstrated that a segment of a porcine coronary artery with denuded and regenerated endothelium exhibited both defective endothelium-dependent relaxation, and hypercontractility of vascular smooth muscle. However, they concluded that the main portion of the alteration of the response was due to enhanced smooth muscle reactivity. In another study, 5-HT and the thromboxane mimetic U46619 produced a marked decrease in blood flow to the retina and choroid in atherosclerotic but not in normal monkeys [87]. Although constrictor responses to serotonin are generally augmented following endothelial removal in healthy arteries [88], several studies have described that the constrictor response to U46619 is independent of the presence of endothelium [89],

suggesting that smooth muscle hypercontractility played a role in the increased vasoconstrictor responses to U46619 in atherosclerotic monkeys. Interestingly, smooth muscle hypercontractility, if existent, may not be an entirely non-specific phenomenon: Indeed, vasoconstrictor responses to angiotensin and (nor)adrenaline were not altered in atherosclerotic monkeys [90]. Also atherosclerosis-related augmented contractile responses to ergonovine have been ascribed to smooth muscle hypersensitivity [91], and Chester and co-workers have described that the contractile response to the 5-HT_1 receptor agonist sumatriptan is augmented in areas adjacent to the atherosclerotic lesion. The response to sumatriptan appeared independent of the presence of endothelium [92].

Alteration of the contraction-relaxation equilibrium due to endothelial regeneration after injury

After balloon catheter-induced injury of the endothelium, endothelial cells rapidly become involved in migration and replication [63], resulting in recovery of endothelium-dependent responses to e.g. bradykinin in denuded porcine coronary artery [93]. However, the number of endothelial cells is increased four weeks after endothelial denudation, and the dilatory response to aggregating platelets is impaired [94]. Like in atherosclerotic blood vessels, the responses to 5-HT and the α_2 receptor agonist UK 14304, are impaired, probably resulting from a defect in pertussis-toxin-sensitive G-proteins. Indeed, the response to e.g. calcium ionophore A23187 and, as mentioned, bradykinin returned to similar levels as before endothelial denudation [80]. In man, a more pronounced contractile response to 5-HT could be observed after coronary angioplasty [95]. Recently, Shibano and Vanhoutte [96] have described that the decreased relaxation to 5-HT in coronary arteries previously denuded of the endothelium, was not observed in pigs treated with a 5-HT_2 receptor antagonist. The exact mechanism for this phenomenon remains unknown. Interestingly, Kalkman and co-workers have shown that the atherosclerosis related supersensitivity in rabbit aorta is due to the unmasking of 5-HT_2 receptors [97].

Alteration of the contraction-relaxation equilibrium due to diet

Despite an obvious correlation between hypercholesterolemia and atherosclerosis, there is evidence that diet itself may affect the contraction-relaxation equilibrium via other mechanisms. Specifically, it was shown that hypercholesterolemia may affect endothelium dependent relaxations, also in the absence of atherosclerosis [98, 99]. In particular oxidized low-density lipoproteins (ox-LDL) were shown to inhibit

endothelium-dependent relaxations, and may thus be involved in impaired relaxations in hypercholesterolemia [100, 101]. Moreover, Boulanger and colleagues found that oxidized low-density lipoproteins induced the expression of preproendothelin mRNA [102]. Lastly, Kaul and co-workers found that, compared to platelets obtained from normal individuals, vasodilator responses to platelets from hypercholesterolemic patients were profoundly impaired, and vasoconstrictor responses were enhanced [103]. The latter phenomenon should be superimposed on an impaired dilatory endothelial function, possibly due to increased serum LDL levels and hypercholesterolemia.

Alteration of the contraction-relaxation equilibrium by physical stimuli

A number of other factors may affect the contraction-relaxation equilibrium in the vascular smooth muscle wall. First, it has been shown that flow and shear stress can induce the release of EDRF, resulting in decreased resistance [104]. Flow-induced vasodilatation was decreased in atherosclerotic blood vessels [105, 106]. Increased transmural pressure, however, results in the release of endothelium derived constricting factors (EDCF). Indeed the rapid increase of transmural pressure of an isolated canine carotid artery resulted in a contractile response. This pressure-induced increase of pressure is followed by a secondary increase of tension only in the presence of endothelium [107]. The nature of the EDCF involved in pressure-induced vasoconstriction remains under debate [104].

2.5 Objectives

The objective of the studies described in this thesis was to investigate possible mechanisms involved in a disturbed equilibrium between relaxation and contraction in human isolated blood vessels. This was done in order to assist the development of novel drugs that may restore this balance, and also in order to understand and improve current drug treatment of ischemic heart disease.

First, we set out to investigate the nature of the receptors involved in the contractile response to 5-hydroxytryptamine (5-HT), using a series of selective 5-HT receptor agonists and antagonists. We investigated 5-HT receptors involved in contractions of the human isolated saphenous vein (Chapter 4), and the human isolated coronary artery

(Chapter 5). In both blood vessels we compared the functionally obtained affinity values of receptor agonists and antagonists to affinity values of these compounds for known 5-HT receptor subtypes, obtained in receptor binding assays. This was done to characterize the receptors mediating contraction as one or two of the known (i.e. cloned) receptor subtypes. In the saphenous vein, we specifically investigated whether altered vascular architecture after being in use as a coronary bypass graft for several years, would alter contractile responses to 5-HT receptor agonists. In the coronary artery, we specifically investigated whether the concentration of 5-HT which induces contraction, would affect the nature of the receptor subtype involved in the contractile response. The latter hypothesis and investigation could offer a possible explanation for the clinical ineffectiveness of the 5-HT_2 receptor antagonist ketanserin in disorders in which 5-HT-induced vasospasm is believed to play a role.

In Chapter 6 we aimed at investigating the mediators involved in the contractile response of the human isolated coronary artery induced by activated platelets. This was done using receptor antagonist for thromboxane and 5-HT, both before and after the platelet-donors had taken low-dose aspirin, in order to understand and possibly improve this common form of treatment for patients with (un)stable angina and myocardial infarction.

In Chapters 7 to 10, we set out to investigate endothelin receptors involved in contractile responses of the coronary artery and saphenous vein. First, we applied quantitative autoradiography to investigate the binding sites in human coronary arteries, using both [^{125}I]-ET-1 and [^{125}I]-sarafotoxin S6b as radioligands (Chapter 7). The involvement of functional correlates of the observed binding sites in contraction was subsequently investigated both in the human isolated saphenous vein (Chapter 8), and in the human isolated coronary artery (Chapter 9). Because a number of observations had been made, that were difficult to interpret in terms of the thus far recognized receptors for endothelin, we investigated whether the type of preparation, or the removal of the endothelium, would affect our observations (Chapter 9). Lastly, we compared our results and those of other investigators to the existing endothelin receptor classification, in order to evaluate the possibility of additional endothelin receptor subtypes, and the general validity of the present classification (Chapter 10).

Throughout the study we attempted to relate contractile responses of various agonists, or platelets, to the functional capacity of the endothelium (measured as relaxation to substance P after precontraction with prostaglandin $F_{2\alpha}$), and to age of the coronary artery donor. In Chapter 6, we histologically analyzed the vascular segments, used to obtain the control response to platelets, in order to relate this contractile response to microscopically observed signs of early atherosclerosis.

2.6 References

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Part 2

5-Hydroxytryptamine and platelets

Chapter 3

5-Hydroxytryptamine: An update on the receptor subtypes and their functional and therapeutical relevance in the cardiovascular system[1]

3.1 The discovery and isolation of 5-hydroxytryptamine (5-HT)[2]

The vasoconstrictor properties of blood serum had been known for over 75 years when serotonin was discovered. Ludwig and Schmidt reported as early as 1868 that perfusion with defibrinated blood increased vascular resistance in dog muscle [3]. However, the underlying mechanism of vasoconstriction was disputed: some favoured involvement of adrenaline (epinephrine) [4], whereas others claimed that an unknown substance was released upon clotting of the blood [5]. In 1911 and 1912, O'Connor showed that serum constricted both the frog perfused vasculature and rabbit isolated intestines, whereas adrenaline caused constriction of the frog blood vessels, but dilatation of rabbit intestines [6, 7]. This indicated that adrenaline was not the constricting factor in serum. The remaining confusion about the chemical properties of the compound was portrayed by Janeway and colleagues who summarized the available information in 1918 by saying that "the substance is or is not dialysable, does or does not resist heat, if [a] protein, is or is not a globulin, [and] if a crystalloid, ... is extracted by various solvents" [8]. Subsequently, Janeway and colleagues showed that the vasoconstrictor compound was localized in platelets and crystalloid in nature; the formation of the compound was dependent on the release from platelets, irrespective of the formation of an actual clot.

In the 1930s, Erspamer and co-workers characterized a substance in rabbit stomach mucosa, which they called enteramine [9]. They found this compound to be present particularly in enterochromaffin cells in the gastroinstestinal mucosa of many species. In an *in vitro* set-up, it stimulated intestinal strips and uterus, as well as molluscan heart. In 1948, Rapport, Green and Page succeeded in isolating the vasoconstrictor compound in serum, and called it 'serotonin' [10]. Soon after that, they concluded that the active structure

[1], For full drug names, see Ref. 30.

[2], Part of the cited papers were taken from Ref. 1 and Ref. 2.

was 5-hydroxytryptamine (5-HT), but they remained faithful to serotonin as a name [11]. Around the same time, Erspamer and co-workers identified their enteramine as being similar to 5-HT or serotonin [12]. The debate on whether the name 5-HT or serotonin should be used, has continued up to the present times. Bacq and colleagues (1951) stated that 5-HT should be preferred since both enteramine and serotonin are incomplete indications of the locations where the substance can be found [13]. Despite this, the name serotonin has remained customary in the United States, where Page and Rapport had made their discoveries. Although perhaps somewhat inaccurate, the name serotonin was acknowledged to be more convenient and accessible when the 5-HT scientific community organized itself as the "Serotonin Club".

3.2 The history of 5-HT receptor classification

'M' and 'D' receptors

Pharmacological investigations with respect to the functional receptors for 5-HT became possible after synthetic 5-HT had been made available [14]. Gaddum and Picarelli observed that 5-HT-induced contractions of the guinea pig ileum could only partially be blocked by morphine or dibenzyline (i.e. phenoxybenzamine) [15]. The combination of dibenzyline and morphine completely blocked the 5-HT-induced responses. In addition, they observed that 5-HT-induced responses in the morphine-pretreated intestine were completely blocked by LSD, 2-bromo-lysergide or dihydroergotamine. However, 5-HT-induced contraction of the dibenzyline-pretreated guinea-pig intestine was completely blocked by atropine and cocaine. Thus, Gaddum and Picarelli concluded that the effect of 5-HT was likely to be mediated via two different receptor mechanisms. The first 5-HT receptor (M receptor, or morphine sensitive receptor) was located on the parasympathetic ganglion. Activation of this receptor would lead to the release of acetylcholine, resulting in contraction. The second 5-HT receptor (D-receptor, or dibenzyline sensitive) was located on the smooth muscle. Activation of this receptor would result directly in contraction of smooth muscle cells [15].

Towards the Bradley et al. classification

Several observations were made that could not be classified as M- or D-receptor-mediated mechanisms. Mianserin, for instance, was a potent antagonist of 5-HT-induced responses in vascular and non-vascular D-receptor preparations. However, mianserin did

not attenuate the vasoconstrictor effect of 5-HT in the canine carotid vascular bed [16]. The use of radioligands to label 5-HT receptors, resulted in rapid further advances towards a new receptor classification. Peroutka and Snyder observed distinct receptors in rat brain membranes, which had either high or low affinity for 5-HT. Only the 5-HT low-affinity site (5-HT_2) had high affinity for spiperone, whereas both sites (5-HT_1 and 5-HT_2) had high affinity for LSD. Affinities for 5-HT_2 sites correlated closely with previously obtained functional affinity parameters for 'D'-receptors. Data obtained with a steadily increasing amount of selective receptor agonists and antagonists resulted in the first consensus classification of 5-HT receptors by Bradley and colleagues in 1986 [18].

Table 1. The Bradley et al. criteria for the classification of functional 5-HT receptors, as proposed in 1986 [18].

Proposed receptor nomenclature	Typical Response	Selective agonist	Selective antagonist	Binding site
5-HT_1-like	Prejunctional inhibition of neuronal transmitter release; smooth muscle relaxation, contraction of some smooth muscle preparations; tachycardia in the cat	5-CT	Methiothepin Methysergide [1]	5-HT_1 [2]
5-HT_2	gastrointestinal and vascular smooth muscle contraction, platelet aggregation neuronal depolarization	-	Ketanserin Cyproheptadine Methysergide	5-HT_2
5-HT_3	Depolarization of peripheral neurones	2-Methyl-5-HT	MDL 72222 Tropisetron Cocaine	None

[1], Weak antagonist or partial agonist at some 5-HT_1-like receptors; [2], Likely to be heterogeneous in nature.

A novel subdivision was agreed upon, consisting of 5-HT_1-like, 5-HT_2, and 5-HT_3 receptors (for criteria, see Table 1). Radioligand binding resulted in a further subdivision of 5-HT_1-like receptors into 5-HT_{1A} and 5-HT_{1B} [19], and later also 5-HT_{1C} [20] and 5-HT_{1D} receptors [21]. An important incentive for this development appeared to be the development of novel subtype selective compounds like the selective 5-HT_{1A} receptor agonist, 8-OH-DPAT [22].

A new framework resulting in a novel 5-HT receptor classification

In 1985, it was reported that tachycardia in the pig after infusion of 5-HT was mediated via a receptor, which was not affected by antagonists for the α, ß, H_1, H_2, 5-HT_1-like, 5-HT_2, or 5-HT_3 receptor. Thus, tachycardia in the pig appeared to mediated via a novel 5-HT receptor [23, 24]. Dumuis and colleagues discovered a novel receptor in the mouse embryo colliculi neurones, positively coupled to adenylyl cyclase, which they tentatively called the 5-HT_4 receptor [25]. Subsequently, it was shown that the 5-HT-induced tachycardia in the pig [26] and positive inotropy in the human isolated atrium [27], and the facilitation of peristalsis in the gastrointestinal tract of the guinea pig, were all mediated via the 5-HT_4 receptor [28].

The early 1990's changed the emphasis from the *functional* characterization of receptors to *structural* characterization. The rapid development in molecular biology techniques resulted in the cloning of new genes encoding receptors of the 5-HT receptor family. On many occasions the function of a newly discovered receptor was unknown. This continuing advancement initiated the development of a new 5-HT receptor classification. In contrast to the early days when receptors could only be characterized from functional stimulation with almost non-selective agonists and antagonists, we were now often confronted with overwhelming information about the functional effects of many selective agonists and antagonists, the exact sequence of the gene encoding for the receptor protein, and about second messenger mechanisms. This large amount of information, taken together with the number of new receptors discovered, required rigorous classification criteria, which had previously been advocated [29] but were first adopted in the novel classification for 5-HT receptors. These three criteria are: i. operational ((ant)agonist-receptor interaction), ii. transductional (second messenger mechanisms), and iii. structural (gene and aminoacid sequence of the receptor peptide) (Table 2). Together, this body of information can provide a finger printing basis for the characterization of a certain receptor. To distinguish receptors that meet all criteria from those of which only a structure has been determined, lower case lettering is applied for the receptors that require

Table 2. Criteria for receptor characterization in general. Modified from Ref. 29-30. Definitive characterization of a receptor (resulting in upper case lettering) ideally requires clarification of all three criteria.

Criterium	Definition of the criterium
1. Operational	
1a. Selective agonists	An agonist with i. high affinity and ii. selectivity relative to other receptors needs to be identified.
1b. Selective antagonists	An antagonist with i. high affinity and ii. selectivity relative to other receptors needs to be identified.
1c. Ligand binding affinity	Dissociation constants for a radioligand should be identified, showing selective, high affinity binding to the receptor.
2. Transductional	Information should be obtained with respect to the transductional mechanism, e.g. coupling to G-proteins or ligand-gated ion-channel: Families of receptors (i.e. receptor-*types*) are usually coupled to the same second messenger mechanism.
3. Structural	Both the amino acid sequence and the nucleotide sequence encoding for the amino-acids should be identified.

additional characterization. Upper case letters will be used when the missing characterization criteria will be fulfilled (for 5-HT receptor classification according to Hoyer and colleagues [30], see Table 3).

When going over the general criteria for the classification of receptors (Table 2) and the 5-HT receptor classification (Ref. 30 and Table 3), a few points need special consideration. First, it can be seen that both 5-HT_1-like receptors and 5-HT_4 receptors have been designated upper-case letters. One of the criteria (elucidation of the receptor structure) had however not been met at the time of publication, but the gene sequence and receptor structure of the 5-HT_4 receptor have recently been disclosed [32]. Moreover, there is some evidence to support the idea that 5-HT_1-like receptors may be heterogeneous, or different in different blood vessels and species (see Table 4).

Table 3. The modern classification of 5-HT receptors: operational and transductional characteristics. Table adapted from Hoyer and colleagues [30]. Data on the structure of the receptor subtypes were omitted.

Receptor Type	Subtype	Location	Response in intact tissue/native cells	Second Messenger	Agonist	Antagonist
$5\text{-}HT_1$	$5\text{-}HT_{1A}$	Neuronal, CNS	Neuronal hyperpolarization, hypotension	Inhibition adenylyl cyclase	8-OH-DPAT buspiron 5-CT	SDZ 216525 WAY 100135 methiothepin
	$5\text{-}HT_{1B}$	CNS and some peripheral nerves	Inhibition of neurotransmitter release	Inhibition adenylyl cyclase	CP 93.129 5-CT	Cyanopindolol SDZ 21009 methiothepin
	$5\text{-}HT_{1D}$	Mainly CNS	Inhibition of neurotransmitter release	Inhibition adenylyl cyclase	Sumatriptan L-694247, 5-CT	GR 127935 metergoline methiothepin
	$5\text{-}ht_{1E}$	CNS	Not known	Inhibition adenylyl cyclase	5-HT	None (methiothepin weak)
	$5\text{-}ht_{1F}$	CNS	Not known	Inhibition adenylyl cyclase	5-HT	None (methiothepin weak)
	$5\text{-}HT_1$-like	Blood vessels	Smooth muscle contraction	Inhibition adenylyl cyclase	Sumatriptan 5-CT	None (methiothepin non-selective)

5-HT_2	5-HT_{2A} [1]	Vascular smooth muscle, platelets, CNS, GI-tract	Vasoconstriction, platelet aggregation, bronchoconstriction	Induction inositol phosphates	α-methyl-5-HT DOI	Ketanserin Ritanserin Pirenperone
	5-HT_{2B} [2]	Rat stomach fundus	Stomach fundus contraction	Induction inositol phosphates	α-methyl-5-HT, DOI	SB 200646
	5-HT_{2C} [3]	CNS	Variety of effects in CNS (e.g. in choroid plexus)	Induction inositol phosphates	α-methyl-5-HT DOI	Mesulergine Ritanserin
5-HT_3		Peripheral and central neurones	Depolarization	Cation channel opening	2-methyl-5-HT *m*-chlorophenylbiguanide	Ondansetron Tropisetron
5-HT_4		GI-tract, CNS, heart, urinary bladder	Induction ACh[4] release in gut, tachycardia	Induction adenylyl cyclase	Metoclopramide renzapride (partial agonist)	GR 113808 SB 204070 Tropisetron
5-ht_{5A} and 5-ht_{5B}		CNS	Not known	Not known	5-HT	Methiothepin
5-ht_6		CNS	Not known	Induction adenylyl cyclase in HEK293 cells	5-HT	Methiothepin
5-ht_7		CNS	Not known	Induction adenylyl cyclase in HeLa/COS cells	5-HT	Methiothepin

[1], previous name: 5-HT_2; [2], previous name: 5-HT_{2B}; [3], previous name: 5-HT_{1C}; [4], ACh, Acetylcholine.

3.3 The effects of the endogenous ligand 5-HT on the cardiovascular system

5-HT can be found in the central nervous system, where it acts as a neurotransmitter, and in several peripheral tissues, such as the gut and platelets in the blood. Most of the 5-HT present in man is synthesized in the enterochromaffin cells in the gastrointestinal tract. Part of this is released into the portal circulation where a substantial fraction of that amount will be stored into platelets. Due to the effective clearance of 5-HT from the plasma by platelets, but particularly by the liver and lungs, the plasma level of 5-HT in the systemic circulation remains extremely low (in nanomolar range) and it is not likely that such concentrations will affect the cardiovascular system (review, see Ref. 33).

Or, as Erspamer put it in 1954 [34]:

> ... We must not forget that to have an insignificant short-lived effect on arterial pressure in human beings at least 60 to 120 µg of 5-HT injected intravenously are required, that is to say an amount of substance equivalent to that contained in 1200 to 2400 ml blood; that the sudden, massive release into the plasma of the entire platelet 5-HT of a dog (7.3 µg/kg) would cause nothing but an insignificant and brief pressure change; and that the subcutaneous injection, in both the dog and the rat, of quantities of 5-HT equivalent to the total content of the substance in the organism has no effect whatever on the blood pressure. ...

Thus, for 5-HT to play a role in the cardiovascular system, it should either act locally at sites where platelets aggregate and release 5-HT in sufficiently high concentrations, or it could play a role via its actions in the central nervous system. Although a tonic presence of 5-HT may play an insignificant role in pathology, this does not rule out the potentially effective access of 5-HT receptors for pharmacological intervention with agonists. Indeed, intravenous administration of *exogenous* 5-HT induced a transient bradycardia and hypotension (Von Bezold-Jarisch reflex), followed by a pressor response, succeeded by a late, but lasting depressor response [35].

Cardiovascular effects of 5-HT mediated via the central nervous system or peripheral neurons

Part of the above mentioned effects after i.v. administration of 5-HT resulted from activation of 5-HT receptors in the central nervous system and peripheral neurons. In particular, the initial bradycardia resulted from stimulation of 5-HT_3 receptors on afferent

cardiac vagal nerve endings. Also the late depressor response may be mediated via an effect on neuronal 5-HT receptors. This response was insensitive to selective 5-HT_2, or 5-HT_3 receptor antagonists, but it was antagonized by the non-selective 5-HT_1-receptor antagonists methysergide and methiothepin [35, 36]. Indeed the i.v. or i.c.v. infusion of agonists for the 5-HT_{1A} receptor resulted in lowering of blood pressure via inhibition of sympatic tone [37]. However, the nature and magnitude of the response after intracerebral administration of the non-selective ligand 5-HT, depended largely on the exact site of application. Different 5-HT receptors in different sites of the brain can mediate either pressor or depressor responses (review, see Ref. 38). 5-HT may also have an effect on postganglionic sympatic neurones, where it may either inhibit the release of noradrenaline via a 5-HT_{1D}-like receptor (in the human saphenous vein: Ref. 39), or induce a tyramine-like effect leading to the release of noradrenaline [40].

5-HT receptors and myocardial contractility

After the discovery that 5-HT_4 receptors mediated a positive chronotropic response in the anaesthetized pig [26, 42] as well as a positive inotropic response in the human isolated atrium [27], it was postulated that 5-HT_4 receptors may provide a possible target for the treatment of heart failure [44]. However, because a positive inotropic response was absent in both human and porcine isolated ventricular trabeculae, the possibility for such treatment was virtually ruled out [45, 46]. Recently, it was suggested that 5-HT_4 receptors may play a role in the precipitation of cardiac arrythmia, and that antagonists at this receptor could serve as anti-arrhythmic drug-treatment [47, 48, 48a]. However, it should be noted that the *in vitro* experiments, leading to this proposition, involved isolated atrial trabeculae paced at less than 1 Hz. The observed irregular contractile responses, although described as extrasystoles and ectopic tachycardia, may also be regarded as abnormal contractions with low contractile force related to a low stimulation frequency. The putative arrhythmias were provoked by 5-HT and attenuated by 5-HT_4 receptor antagonists. Certainly, this interesting phenomenon appears mediated via 5-HT_4 receptors. However, it is quite likely that the observations are not analogous to cardiac arrhythmia, which involves a complex of spontaneous rhythmical discharge and conduction in the sino-atrial node and Purkinje fibres. All of these are virtually absent in stimulated isolated trabeculae. Therefore, there is yet little evidence that the 5-HT_4 receptor is involved in arrhythmia. In particular, *in vivo* animal studies or human clinical trials are necessary to provide further clues about the use of 5-HT_4 receptor antagonists as a novel approach in anti-arrhythmic treatment.

5-HT receptors in blood vessels

5-HT induced endothelium-dependent relaxation in porcine [49] and canine [50] coronary arteries. In porcine coronary arteries, relaxation was shown to be mediated via 5-HT_{1D}-like receptors [51]. Alteration of vascular architecture due to experimentally-induced atherosclerosis, or endothelial denudation after balloon angioplasty, were shown to result in altered responses to 5-HT, both *in vivo* and *in vitro*. *In vivo*, endothelial denudation or diet-induced atherosclerosis resulted in increased vasoconstriction after infusion of 5-HT [52, 53]. *In vitro*, the 5-HT-induced relaxant response was attenuated after balloon denudation of the porcine coronary artery [49, 54].

5-HT, infused in the coronary artery of healthy humans, induced vasodilatation, which was potentiated by pretreatment with ketanserin, a 5-HT_2 receptor antagonist. In patients with Prinzmetal's angina pectoris or atherosclerotic coronary artery disease, intracoronary infusion of 5-HT induced constriction [55, 56]. This response was completely attenuated by ketanserin in proximal coronary arteries, and partially attenuated in distal coronary arteries [57]. Moreover, the constrictor response induced by 5-HT was more pronounced in coronary arteries after a single percutaneous transluminal angioplasty (PTCA) [58]. In human coronary arteries *in vitro*, the contractile response mediated via 5-HT_1-like receptors was relatively more pronounced in the segment distal to an occluded coronary artery segment [59]. Thus, also in man, the response to 5-HT may be altered in diseased blood vessels. It may be concluded that 5-HT induces vasodilatation of human coronary arteries *in vivo* via a ketanserin-insensitive (presumably 5-HT_1) receptor. Moreover, 5-HT induces vasoconstriction via ketanserin-sensitive (5-HT_2) receptors, as well as via a ketanserin-insensitive, sumatriptan sensitive (5-HT_1) receptor.

In addition to alteration of the response of the blood vessels to 5-HT, an increased 5-HT plasma concentration was observed in patients with angina and complex coronary artery lesions [60]. Moreover, the coronary sinus plasma of patients with coronary artery stenosis, induced vasoconstriction of canine coronary artery rings, correlating with the extent and severity of coronary artery narrowing. The coronary sinus plasma obtained from patients with normal coronary arteries did not elicit a contractile response [61]. In another study, vasoconstriction developed after undergoing angioplasty, correlating significantly with the concentration of 5-HT measured in the coronary sinus plasma [62].

Table 4. Examples of operational discrepancies of a selection of vascular 5-HT_{1D}-like receptors. The differences may reflect different 5-$HT_{1(D)}$-like receptors. Values between brackets refer to pA_2/pK_B values. Antagonists are tested against receptor agonists at 5-HT_1-like receptors.

Species	Localization	Agonist / Antagonist actions
Dog	Saphenous vein [64]	Methiothepin relatively potent (8.3-8.6) Metergoline relatively weak (6.4) Rauwolscine inactive (1 μM) Ketanserin inactive (1 μM)
Rabbit	Saphenous vein [65, 66]	Methiothepin relatively potent (9.5 [65]; 8.3 [66]) Cyanopindolol inactive (1 μM) [66] Ketanserin active (7.5 [66]; ±6.7 [65])
	Renal artery [67]	Ketanserin active (6.6) Methiothepin relatively potent (8.6) Metergoline inactive (0.1 μM)
Guinea-pig	Iliac artery [68]	Cyanopindolol and metergoline low affinity *agonists*: pD_2 6.0 and 5.8, resp. As antagonist: metergoline relatively weak (7.0) Ketanserin active (6.2) Methiothepin relatively potent (8.4)
Human	Basilar artery [69]	Methiothepin potent (8.8) Ketanserin and cyanopindolol inactive (0.1 μM)
	Pial arteriole [70]	Methiothepin relatively potent (8.6*) Metergoline relatively weak (6.9*) Ketanserin inactive (1 μM)
	Saphenous vein [71]	Ketanserin inactive (1 μM) Metergoline relatively potent (6.9-7.3) Cyanopindolol (6.5) & rauwolscine (6.7) active Methiothepin relatively weak (7.1-7.9)

*, Slope of the regression line in the Schild-plot differed significantly from unity.

Contraction of vascular smooth muscle is often mediated by 5-HT_2 receptors, except in the cranial vasculature, where 5-HT_1-like receptors are involved [63]. However, this concept of peripheral 5-HT_2 receptor-mediated vasoconstriction vs. cranial 5-HT_1-like receptor-mediated vasoconstriction did not entirely apply to the human vasculature. Only the human femoral vein [74] contracted solely via 5-HT_2 receptors. 5-HT_1-like receptors played an additional role in the 5-HT-induced contractile response of other investigated human blood vessels.

The nature of the 5-HT_1-like receptor is still under considerable debate. Recent evidence suggests that 5-$HT_{1D\alpha}$ [75] or 5-$HT_{1D\beta}$ [76, 77] receptors may be present in the coronary artery of dogs and man, respectively. Also in pial arterioles it was suggested that the receptor mediating contraction is similar to the 5-$HT_{1D\beta}$ receptor [72]. However, it should be emphasized that no selective ligands with high affinity are available. Both in the canine coronary artery and in the pial arteriole the receptor was shown via PCR, which supported the presence of mRNA for the respective receptors but provided no evidence on their involvement in the contractile responses to 5-HT. In addition, there is evidence to support heterogeneity of 5-HT_1 receptors that mediate the contractile response to 5-HT. For instance, the potency of methiothepin as compared to metergoline as antagonists against 5-HT_1 receptor agonism, as well as the potency of ketanserin and cyanopindolol may vary considerably. Discrepancies exist both between the vascular beds within species, as well as between species (Table 4).

3.4 Therapeutic applications for 5-HT receptor ligands in the cardiovascular system

Hypertension

Inhibition of peripheral 5-HT receptors has been suggested as a mode of treatment of hypertension, and ketanserin is marketed as an antihypertensive drug with 5-HT_2 receptor antagonistic effects. Several lines of evidence dispute this mechanism of action of ketanserin. First, it should be remembered that endogenous 5-HT exists in the plasma at clearly lower concentrations than needed to evoke generalized pressor responses [33]. High levels may be reached locally, following platelet aggregation, but this remains insufficient to evoke systemic responses, albeit that local vasospasm may occur. Secondly, it was shown that ritanserin -also a 5-HT_2 receptor antagonist, but devoid of the α-adrenoceptor antagonist effect of ketanserin- did not decrease blood pressure [78, 79]. Thus, it was concluded that mainly the α-adrenergic receptor antagonism was responsible for the

antihypertensive effect. In addition, it was suggested that central 5-HT_2 receptors may play a role in the anti-hypertensive effects of ketanserin [80, 81], but the significance of this phenomenon in hypertensive individuals remains unclear.

Urapidil is marketed as antihypertensive drug with α_1 receptor antagonist effects [82]. However, urapidil also displayed antihypertensive activity when administered centrally. It was established in animal experiments that this was mediated via central 5-HT_{1A} receptors for which urapidil has substantial affinity. The precise relevance of the 5-HT_{1A} receptor agonism in the hypotensive effect in man is yet unclear, but treatment with urapidil was devoid of reflex tachycardia, which may be due to urapidil's effect on central 5-HT_{1A} receptors, resulting in sympathoinhibition [83].

Flesinoxan is a 5-HT_{1A} receptor agonist without α-adrenoceptor antagonist activity. Based on subchronical studies in spontaneously hypertensive rats, 5-HT_{1A} receptor-mediated sympathoinhibition in the central nervous system was believed to be an interesting alternative approach in the treatment of hypertension [37, 84]. However, despite promising results with single doses of flesinoxan in patients with mild hypertension [85], patients developed tolerance to the antihypertensive effect of flesinoxan during a double blind placebo-controlled clinical trial [37]. Flesinoxan is presently under investigation for the treatment of anxiety, depression, or obsessive compulsive disorders.

Migraine

Considerable debate still exists on the pathophysiological mechanism of migraine, and on the mode of action of anti-migraine drugs. Some researchers advocate the assumption that migrainous pain is induced by pulsations in dilated cranial blood vessels, caused by yet unknown migraine triggers. This theory proposes that anti-migraine drugs like sumatriptan constrict the cranial blood vessels to normal diameter and pulsation, thereby alleviating pain [86-88]. Others support the notion that yet unknown triggers of migraine induce neurogenic-inflammation. These triggers result in the release of a number of mediators (presumably vasoactive neuropeptides like CGRP and perhaps substance P), which increase vascular permeability, leading to vasodilatation and stimulation of sensory nerve fibers. Anti-migraine drugs, like sumatriptan, suppress the migraine attack by activating presynaptic 5-HT_1-like receptors, resulting in a decreased release of the inflammatory peptides, a reduced vascular permeability and vascular diameter, which would lead to cessation of pain [89]. It is not unlikely that both theories may ultimately prove to be correct. However, from a drug developer's point of view, it is extremely important to establish the precise nature of mechanisms and receptor subtypes involved in both theories. It may become possible to develop compounds selective for presynaptic 5-HT_1-

like receptors that do not induce constriction of blood vessels. Although it is not certain that such 'anti-neurogenic-inflammation drugs' would be sufficiently effective in the treatment of migraine, they may prove to be devoid of the vasoconstrictor side effects of the current generation of anti-migraine drugs. Indeed, sumatriptan as well as ergotamine and dihydroergotamine (DHE) cause coronary vasoconstriction and cardiac ischemia in some patients [90-92].

Restenosis following percutaneous transluminal angioplasty (PTCA)

Although PTCA is a relatively safe and effective treatment for luminal occlusion of the coronary arteries, recurrence of stenosis (restenosis) occurs in 20-40% of patients [93]. Restenosis following PTCA should be divided into acute restenosis (i.e. within 24 hours after the procedure) and chronic restenosis (i.e. peak at 6-8 weeks, up to 6 months) [94, 95]. Acute restenosis occurred in 2-11% of patients undergoing PTCA and was the main cause of in-hospital morbidity and mortality [95]. Balloon angioplasty may lead to impaired endothelium- dependent relaxation in response to administration of 5-HT in the porcine isolated coronary artery [49], whereas in man the contractile response to exogenously administered 5-HT was increased [58]. It was also shown that endogenous 5-HT was released into the coronary circulation during angioplasty, which may contribute to the vasoconstriction in segments distal to the site of dilatation, observed immediately after angioplasty. Furthermore, it was shown that this post-angioplasty vasoconstriction could be attenuated by ketanserin [62]. Indeed, ketanserin reduced the frequency of early restenosis [96]. 5-HT was also shown to induce the mitogenesis of vascular smooth muscle cells *in vitro* [97], which could be attenuated by ketanserin, indicating involvement of 5-HT_2 receptors [98]. However, ketanserin was ineffective in the prevention of chronic restenosis [96, 99], and it may therefore be speculated that *in vivo* the 5-HT_2 receptor contributes only little to the complex process of neointima formation. Interestingly, 10 μM ketanserin did not completely block 5-HT-induced vascular smooth muscle proliferation *in vitro* [98], and 5-HT_{1D}-like receptors have been suggested to be involved in the mitogenic effects of 5-HT as well [100]. Recently, 5-HT_{1D} receptors were shown to be involved in cell proliferation in human small cell lung carcinoma [101]. Further studies are required to establish the potential for a mixed 5-HT_{1D}-like / 5-HT_2 receptor antagonist against early and late restenosis after PTCA.

Vasospasm

Clinical trials have been conducted to investigate the efficacy of 5-HT receptor antagonists in diseases involving peripheral vasospasm. Atherosclerosis [102] and endothelial damage [62], as well as potentiation by alternative vasoconstrictor compounds [103], and enhanced activation of platelets [104] may all result in abnormal vasoconstriction induced by 5-HT released from aggregating platelets. In addition, coronary artery disease is known to be associated with increased levels of serum 5-HT [60, 61]. Therefore, it was rather disappointing that no beneficial results of treatment with ketanserin were observed in five patients with Prinzmetal's angina [105].

5-HT has also been implicated in the pathophysiology of Raynaud's phenomenon, which is a syndrome of cold- or stress-induced vasospasm in digits, resulting in local hypoxia and paleness [106]. 5-HT induced potent vasoconstriction of digital vasculature [107, 108], and platelet aggregation may be accelerated in patients with Raynaud's phenomenon [109]. Indeed, ketanserin has sometimes been shown to be effective in treating Raynaud's phenomenon [110, 111], but like in hypertension, its mechanism of action via 5-HT_2 receptors was questioned [112]. A recent study observed no alterations in plasma levels of 5-HT, even during vasospastic attacks [113], and it is quite likely that the beneficial effects of ketanserin in Raynaud's phenomenon can be attributed to the α-antagonist activity of the compound [33].

3.5 Conclusions

The field of 5-HT receptors, and the role in cardiovascular and non-cardiovascular diseases has seen tremendous developments over recent years. In particular the area of 5-HT receptor classification has developed itself as an example for the classification of receptors of other endogenous mediators. Also the progress in the understanding and treatment of migraine (selective 5-HT_{1D}-like receptor agonists), chemostatic-induced nausea (5-HT_3 receptor antagonists), and a variety of psychiatric disorders (depression, anxiety, obsessive compulsive disorder: 5-HT_{1A} receptor agonists; 5-HT uptake inhibitors), has been impressive, and is still in full swing.

In cardiovascular disorders, in which a pathophysiological tone of 5-HT was believed to play a role (like hypertension, restenosis and vasospastic angina), 5-HT receptor antagonists have thus far not been proven very effective. This could be due to overestimation of the relative involvement of 5-HT in the disease. Indeed, the renin-angiotensin system and cathecholamines appear to play a more significant role, as can be

judged from the clinical efficacy of inhibitors of these endogenous compounds. Also in the chronic development of restenosis, growth factors like platelet-derived growth factor and endothelins could play a more significant role than 5-HT. However, it must be kept in mind that the 5-HT receptor antagonist used in clinical trials, was an antagonist selective for 5-HT_2 receptors. Thus, the effect at other 5-HT receptors was preserved, and this may be of significance when considering additional involvement of a 5-HT_1-like receptor in vascular constriction, and perhaps also in vascular smooth muscle mitogenesis.

3.6 References

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Chapter 4

5-Hydroxytryptamine-induced contractions of the human isolated saphenous vein: Involvement of 5-HT_2 and 5-HT_{1D}-like receptors, and a comparison with grafted veins [*, 1]

Summary - The receptors mediating the contractile effect of 5-hydroxytryptamine (5-HT) in the human isolated saphenous vein, obtained from 42 patients undergoing coronary bypass surgery, have been further characterized using a number of 5-HT-related drugs. The rank order of agonist potency was 5-CT ≈ 5-HT > methysergide ≈ sumatriptan ≈ α-methyl-5-HT ≈ RU 24969 ≈ DOI > 2-methyl-5-HT > 8-OH-DPAT. Flesinoxan was inactive as an agonist. Ketanserin (1 μmol/l) hardly affected sumatriptan-induced contractions but it caused a rightward shift of the upper part of the concentration-response curve of 5-HT and 5-CT. The same concentration of ketanserin caused a parallel rightward shift of the concentration-response curves of α-methyl-5-HT and DOI with pK_B values of 7.1 and 7.1, respectively. The responses to sumatriptan were antagonized by methiothepin (0.1 μmol/l), metergoline (0.1 and 1 μmol/l), rauwolscine (1 μmol/l) and cyanopindolol (1 μmol/l); the calculated pK_B values were 7.3, 6.9, 7.3, 6.7 and 6.5, respectively. Contractions to 5-HT were antagonized by methysergide (1 μmol/l), methiothepin (0.1 μmol/l;

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[1], Novel information on 5-HT receptors has become available since the experiments in the present chapter were performed and analyzed. The 5-HT_{1C} receptor was redefined as 5-HT_{2C} receptor. Recently identified 5-HT receptor subtypes with a 5-HT_{1D}-like receptor profile appear either positively coupled to adenylyl cyclase (5-HT_6, 5-HT_7), or do not match the presently obtained profile for 5-HT_1 receptor agonists, or antagonists against sumatriptan. The pharmacological distinction between the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptor subtypes can not be made in functional studies at present, due to lack of selective receptor agonists and antagonists (See Ref. 47).

pK_B=7.1), ICS 205-930 (1 μmol/l; pK_B=5.9) and flesinoxan (30 μmol/l; pK_B=5.3). Remarkably, the contractions elicited by 2-methyl-5-HT were not attenuated by ICS 205-930, but were antagonized by methiothepin (0.1 μmol/l) and, more markedly, by ketanserin (1 μmol/l).

There was a high correlation between the functional pD_2 values of 5-HT_1-like receptor agonists (5-CT, 5-HT, methysergide, sumatriptan, RU 24969 and 8-OH-DPAT) and their reported binding affinities for the 5-HT_{1D} receptor in human or calf brain membranes. Such a correlation for the antagonism of sumatriptan-induced responses was less marked than for the agonists, but of the 5-HT_1-like receptor subtypes it was the highest for the 5-HT_{1D} receptor identified in human or calf brain membranes.

In 3 patients, undergoing heart transplantation, saphenous vein which had previously functioned as a graft for 6-11 years, was dissected out from the heart. Though the contractions to potassium were significantly smaller in the grafted veins, the pD_2 and E_{MAX} values (calculated as percentage of potassium-induced contractions) for 5-HT and sumatriptan were similar to those found in the veins obtained directly from the lower leg.

It is concluded that contractions in the human isolated saphenous vein induced by 5-HT are mediated by 5-HT_2 receptors as well as by a 5-HT_1-like receptor resembling the 5-HT_{1D} subtype found in brain membranes. It is also to be noted that 2-methyl-5-HT, considered selective for the 5-HT_3 receptor, contracts the saphenous vein mainly via 5-HT_2 receptors.

4.1 Introduction

Saphenous vein and internal mammary artery are frequently used as a graft in coronary bypass surgery. Several differences between these two vessels have been found that could possibly account for the superior patency of mammary artery grafts [1, 2]. Recently, Yang and co-workers [3] reported that, when precontracted with noradrenaline, the human isolated saphenous vein contracts, but the human isolated internal mammary artery relaxes to platelets. Platelet-derived ADP caused the mammary artery to relax while contractions in the saphenous vein were shown to be mediated by thromboxane A_2 and 5-HT. The 5-HT-induced saphenous vein contractions are sensitive to ketanserin and spiperone, both 5-HT_2 receptor antagonists. This ketanserin-sensitive receptor is activated

mainly at high concentrations of 5-HT, while lower concentrations seem to activate a yohimbine- and methiothepin-sensitive 5-HT receptor [4-7]. The present study was undertaken to further characterize the 5-HT receptors mediating contractions in the human saphenous vein. In addition, we also had the opportunity to compare the responses mediated by 5-HT receptors in the vessels which had functioned as coronary bypass grafts for a number of years. A part of the results of this investigation was presented to the British Pharmacological Society [8].

4.2 Materials and methods

Tissue preparation

Leftover human saphenous vein was obtained intra-operatively during bypass surgery from 42 patients (29 males and 13 females; age: 44 to 77 years). The tissue was immediately placed in a cold, oxygenated Krebs bicarbonate solution of the following composition (mmol/l): sodium chloride, 118; potassium chloride, 4.7; calcium chloride, 2.5; magnesium sulphate, 1.2; potassium dihydrogen phosphate, 1.2; sodium bicarbonate, 25; and glucose, 8.3; pH 7.4. The vein was brought to the laboratory within 30 min of removal and cleaned of adhesive fat and connective tissue. In addition, saphenous vein could also be obtained from three male patients (aged 46, 48 and 61 years) with progressive ischemic heart disease, undergoing cardiac transplantation. These patients had previously undergone coronary bypass surgery where saphenous vein had been used as a graft for a period of 6, 7 and 11 years, respectively, prior to transplantation. The vein was collected from these hearts within 45 minutes of cardiectomy. The vessels were cut into rings of approximately 4-5 mm of length and suspended on stainless steel hooks in 8 ml organ baths containing the Krebs' bicarbonate solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37° C. The tissues were allowed to equilibrate for at least 30 min and washed every 10 min. Changes in isometric tension were recorded with a Harvard isometric transducer. Preparations were then stretched to the optimum point of the length-tension relation, as determined by tension developed to 30 mmol/l K^+. The rings were then left to equilibrate for another 30 min after which the actual experiment began. Endothelial integrity was not assessed in these experiments, since the presence of endothelium does not influence 5-HT-induced contractions in this vessel [9].

Determination of agonist potency

A cumulative concentration-response curve to 5-HT was obtained on each preparation until a maximum response (E_{MAX}) was obtained. After frequent washing the tissue was left to equilibrate for at least 30 min. When the baseline tone was reached, the tissue was exposed to either 5-HT again or to another test agonist. In each vessel segment, the second 5-HT concentration-response curve or the *only* concentration-response curve to the other agonists was expressed as percentage of the E_{MAX} of the first 5-HT curve.

To determine agonist potency in saphenous vein grafts that had been in situ for some years, a single concentration effect curve was obtained with either 5-HT or sumatriptan. These responses were expressed as a percentage of potassium (30 mmol/l)-induced contractions in the grafted veins. The response to potassium (30 mmol/l), and the E_{MAX} and pD_2 values (see below) of 5-HT and sumatriptan were compared to the respective response in the ungrafted saphenous veins.

Determination of antagonist potency

A concentration-response curve to 5-HT was made and the preparation was washed as described above. About 30 min later, a second cumulative concentration-response curve was obtained with either 5-HT or another agonist, without or after incubation (30 min) with a certain concentration of an antagonist. The second curve without antagonist, mostly obtained in parallel, served as a control for the curve in the presence of the antagonist.

Analysis of data

All curves were analyzed by means of a computerized curve fitting technique [10] to obtain E_{MAX} (maximal response) and pD_2 (-Log of the molar concentration of an agonist eliciting half maximal effect) values, which were averaged for the respective agonists. For antagonists approximate pK_B values were calculated, assuming the nature of antagonism to be competitive, by the equation described by Furchgott in 1972 [11]:

$$pK_B = -Log[B] + Log\{([A_2]/[A_1])-1\},$$

where [B] is the antagonist concentration and $[A_1]$ and $[A_2]$ are, respectively, the equi-effective molar concentrations of the agonist needed to elicit half maximal effect in the absence and presence of [B]. For calculation of pK_B values, only paired experiments were taken into account, unless mentioned otherwise. All data are presented as mean ± SEM. A correlation coefficient was calculated according to Pearson. To test the hypothesis of a straight line underlying the points in Figure 6, lack of fit statistics [12] was performed using α=0.05 as a critical value. Student's *t*-test for unpaired data was used for

comparison of mean values. A p-value of ≤0.05 was assumed to denote a significant difference.

Compounds

The following drugs were used in this study: 5-carboxamidotryptamine maleate (5-CT; gift: Glaxo, Ware, UK), (±)cyanopindolol (gift: Sandoz AG, Basel, Switzerland), 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI; Research Biochemicals Inc., Natic, Ma., USA), flesinoxan hydrochloride (gift: Duphar B.V., Weesp, The Netherlands), 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT; gift: Merrel Dow Research Institute, Strassbourg, France), 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma Chemical Co., St. Louis, Mo., USA), 3α-tropanyl-1H-indole-3-carboxylate (ICS 205-930; gift: Sandoz AG, Basel, Switzerland), ketanserin tartrate (gift: Janssen Pharmaceutica, Beerse, Belgium), metergoline (gift: Farmitalia, Milan, Italy), methiothepin maleate (gift: Hoffman La Roche B.V., Mijdrecht, The Netherlands), 5-methoxy-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole succinate (RU 24969; gift: Roussel Laboratories, Hoevelaken, The Netherlands), 2-methyl-5-hydroxytryptamine maleate (2-methyl--5-HT; gift: Glaxo, Ware, UK), α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT; gift: Glaxo, Ware, UK), rauwolscine hydrochloride (Fluka AG, Buchs, Switzerland) and sumatriptan (gift: Glaxo, Ware, UK). We appreciate the generosity of the companies in providing the compounds.

4.3 Results

Effect of agonists

The concentration-response curves obtained on the human isolated saphenous vein with the different agonists and the derived values of pD_2 and E_{MAX} are shown in Figure 1 and Table 1, respectively. It is to be noted that contractions in the second 5-HT concentration-response curve were only slightly less than in the first 5-HT curve. All other agonists, except flesinoxan, were found to contract the saphenous vein in a concentration-dependent manner. The rank order of potency was: 5-CT ≈ 5-HT > methysergide ≈ sumatriptan ≈ α-methyl-5-HT ≈ RU 24969 ≈ DOI > 2-methyl-5-HT > 8-OH-DPAT.

Table 1. Agonist potency and maximal effect in the human isolated saphenous vein. pD_2 is expressed as -log(EC_{50}) and E_{MAX} is expressed as a percentage of the maximal 5-HT contraction in the first concentration-response curve

Agonist	n	pD_2 (± S.E.M.)	E_{MAX} (± S.E.M.)
5-CT	5	7.1 (0.2)	89 (4)
5-HT, first	18	7.0 (0.1)	100 (0)
5-HT, second	18	6.8 (0.1)	93 (3)
Methysergide	4	6.3 (0.4)	16 (3)
Sumatriptan	18	6.1 (0.1)	37 (4)
α-Methyl-5-HT	3	6.0 (0.1)	94 (3)
RU 24969	5	5.8 (0.2)	62 (6)
DOI	8	5.7 (0.3)	57 (7)
2-Methyl-5-HT	7	4.8 (0.2)	49 (5)
8-OH-DPAT [a]	8	< 4.6 (0.2)	> 52 (10)
Flesinoxan	3	inactive	inactive

[a], The concentration-response curve did not achieve a plateau even at the highest concentrations studied (10^{-4} mol/l); n, Number of observations.

Table 2. Comparison of the effect (developed tension in g) of potassium (30 mmol/l), and the pD_2 (expressed as -Log[EC_{50}]) and E_{MAX} (expressed as a percentage of the potassium-induced contraction) values of 5-HT and sumatriptan in ungrafted saphenous veins, obtained directly from the lower leg (n=18), and in those which had been used as a coronary bypass graft (n=3)

	Ungrafted vein (± S.E.M.)	Bypass graft (± S.E.M.)
Effect 30 mmol/l potassium	5.5 (0.8)	0.5 (0.9) *
pD_2 5-HT	7.0 (0.1)	6.7 (0.5)
E_{MAX} 5-HT	181 (13)	242 (23)
pD_2 sumatriptan	6.1 (0.1)	5.8 (0.4)
E_{MAX} sumatriptan	59 (8)	70 (15)

*, $p<0.05$, when compared to data in ungrafted vein.

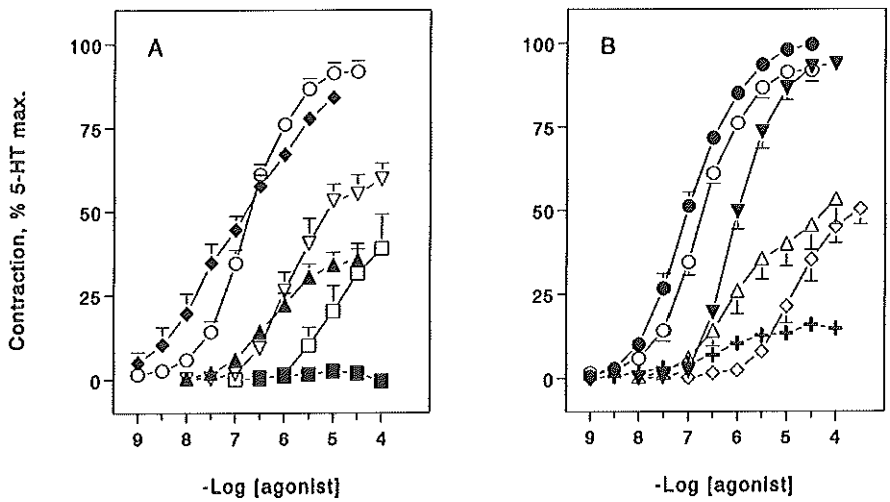

Figure 1. Contractions of the human isolated saphenous vein to 5-HT receptor agonists. Graph A: ○, 5-HT (2nd curve); ◆, 5-CT; ▽, RU 24969; ▲, sumatriptan; □, 8-OH-DPAT and ■, flesinoxan. Graph B: ●, 5-HT (1st curve); ○, 5-HT (2nd curve); ▼, α-methyl-5-HT; △, DOI; ◊, 2-methyl-5-HT and +, methysergide. Agonist concentration refers to mol/l.

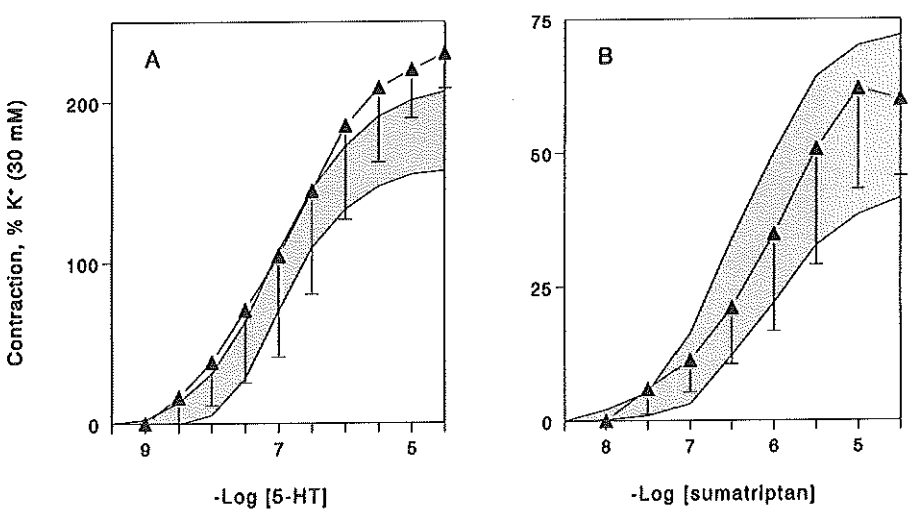

Figure 2. Contractions to 5-HT (A) and sumatriptan (B) in previously grafted saphenous veins (n=3) against a shaded area of the 95% confidence interval of data obtained in saphenous veins obtained directly from the lower leg (n=18). Agonist concentration refers to mol/l. Contractions expressed as a percentage of potassium (30 mmol/l)-induced contractions.

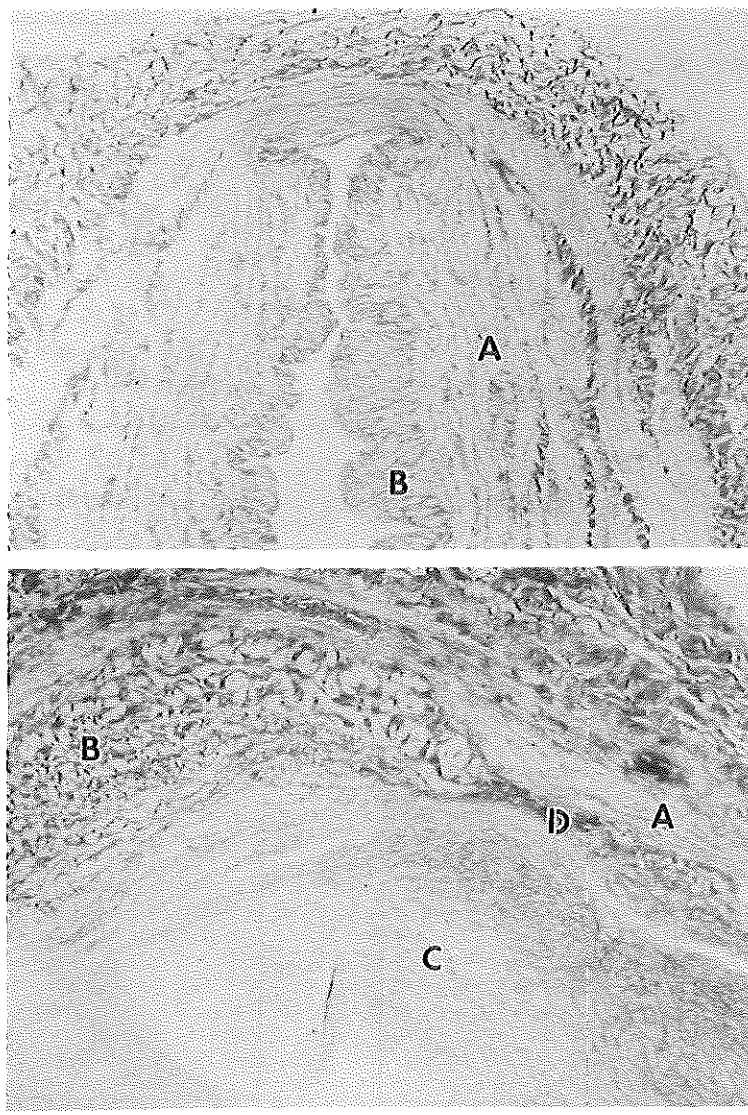

Figure 3. Hematoxylin-eosin stained segments of ungrafted (upper picture) and grafted saphenous vein segments. In the media of the ungrafted vein, a regular organization of circular (A) and longitudinal (B) smooth muscle cells can be observed. Intimal hyperplasia is virtually absent. In the grafted vein, pronounced luminal stenosis was observed due to intimal hyperplasia (C), consisting of myofibroblasts in extracellular matrix. The longitudinal and circular smooth muscle layers are partially reduced, and may be absent in some areas (D).

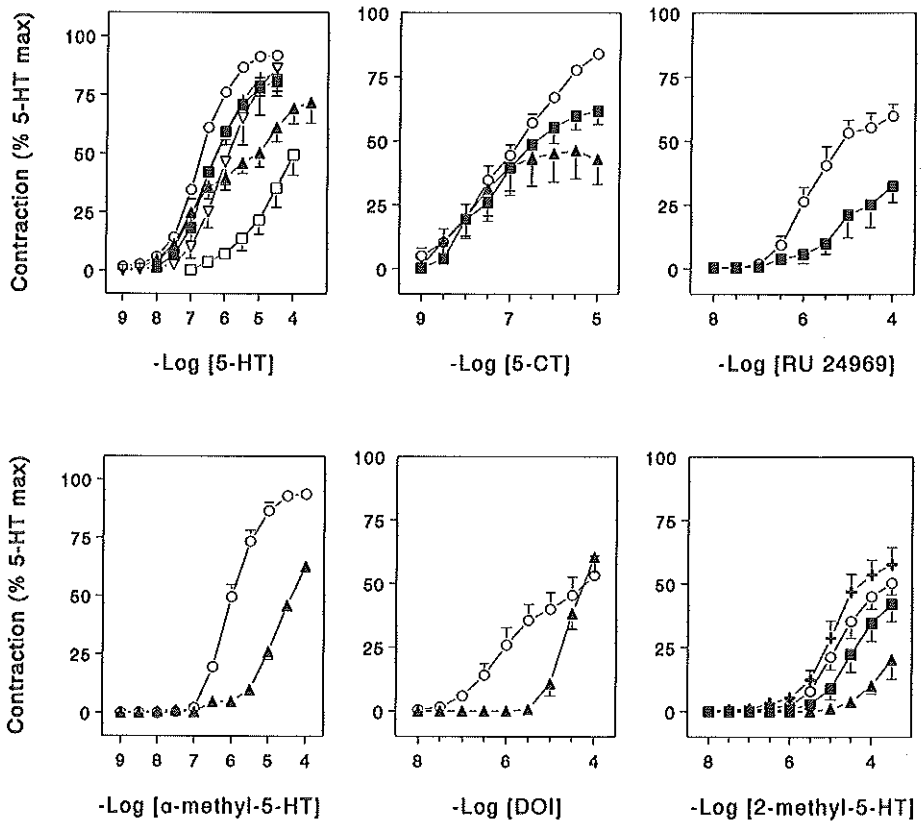

Figure 4. Contractions of the human isolated saphenous vein to several 5-HT receptor agonists in the absence (control, ○) or presence of ketanserin (1 μmol/l, ▲), ICS 205-930 (1 μmol/l, +; hidden behind methiothepin curve), methiothepin (0.1 μmol/l, ■), methysergide (1 μmol/l, □) and/or flesinoxan (30 μmol/l, ▽). Agonist concentration refers to mol/l. pK_B values are mentioned in Table 3. Number of segments tested: 5-HT (control, 18; ICS 205-930, 5; flesinoxan, 4; ketanserin, 5; methiothepin, 5 and methysergide, 3), 5-CT (control, 5; ketanserin, 5 and methiothepin, 6), RU 24969 (control, 5 and methiothepin, 5), α-methyl-5-HT (control, 3 and ketanserin, 3), DOI (control, 7 and ketanserin, 5) and 2-methyl-5-HT (control, 7; ICS 205-930, 5; ketanserin, 5 and methiothepin, 5). In cases where the number of experiments mentioned here does not correspond to the number mentioned in Table 3, unpaired experiments are displayed also

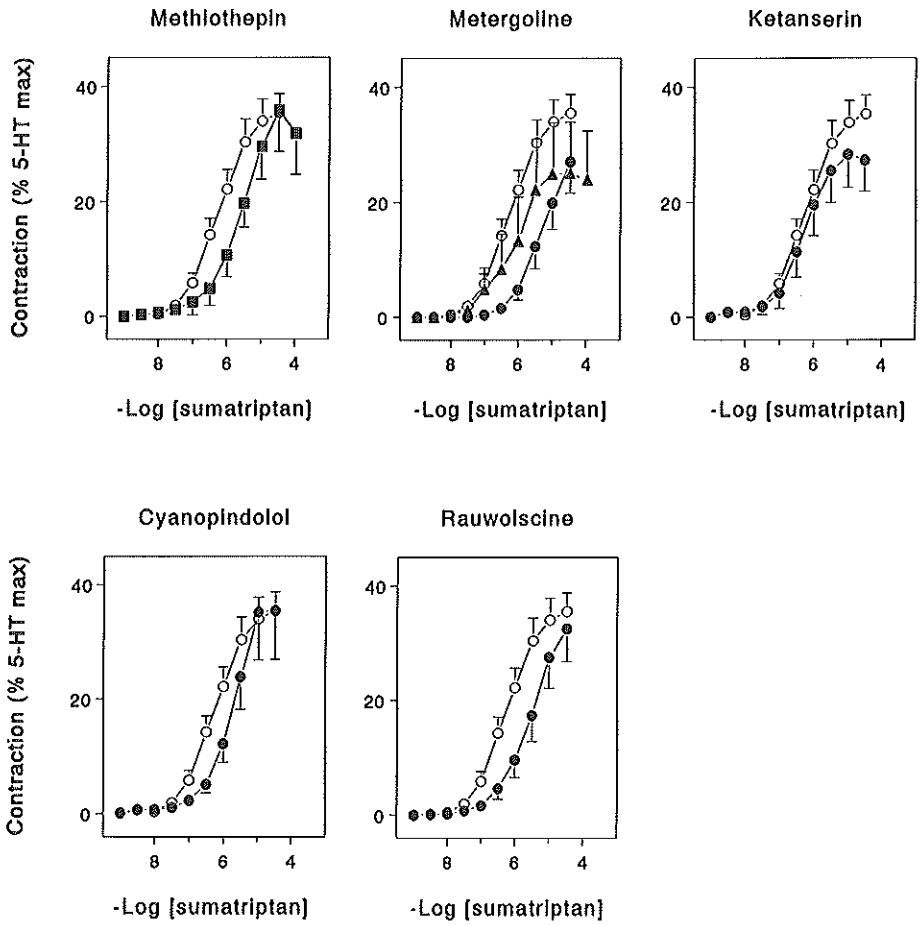

Figure 5. Contractions of the human isolated saphenous vein to sumatriptan in the absence (control, ○) or presence of antagonists (0.1 μmol/l, ▲ and 1 μmol/l, ●). Agonist concentration refers to mol/l. Number of segments tested: control, 18; methiothepin, 8; ketanserin, 8; metergoline (1 μmol/l), 7; metergoline (0.1 μmol/l), 6; cyanopindolol, 6 and rauwolscine, 8. pK_B values, calculated from paired experiments only, can be found in Table 3. In cases where the number of experiments mentioned here does not correspond to the number mentioned in Table 3, unpaired experiments are displayed also.

As shown in Table 2, the exposure to potassium (30 mmol/l) caused contractions of the saphenous veins that had been used as a graft, but the tension generated in these vessels was only about 10% of that observed in the ungrafted veins. Though the responses to both 5-HT and sumatriptan in the grafted vessels were also diminished, the respective E_{MAX} values, when expressed as a percentage of potassium (30 mmol/l)-induced contraction, as well as the pD_2 values were similar to those in the non-grafted veins (Table 2; Figure 2).

Effect of antagonists

The effects of ketanserin (1 μmol/l), methiothepin (0.1 μmol/l), methysergide (1 μmol/l), flesinoxan (30 μmol/l) and ICS 205-930 (1 μmol/l) were studied against the responses to at least one of the following agonists: 5-HT, 5-CT, RU 24969, α-methyl-5-HT, DOI and 2-methyl-5-HT (Figure 4). The calculated pK_B values are shown in Table 3. The responses to 5-HT were antagonized by methysergide, ketanserin and methiothepin (pK_B=7.1±0.4), but also to some extent by ICS 205-930 (pK_B=5.9±0.1) and flesinoxan (pK_B=5.3±0.2). The pK_B values for methysergide (concentration-response curve did not reach plateau) and ketanserin (biphasic concentration-response curve) were not calculated (see Table 3). The antagonism by ketanserin was more marked against contractions due to higher concentrations of 5-HT. The effects of low concentrations of 5-CT were little affected by ketanserin (1 μmol/l) or methiothepin (0.1 μmol/l), but those of high concentrations (≥0.1 μmol/l) were attenuated by both methiothepin and ketanserin. The contractile effects of RU 24969 were antagonized by methiothepin (0.1 μmol/l). Contractions to α-methyl-5-HT and DOI were attenuated by ketanserin (1 μmol/l), while those to 2-methyl-5-HT, being unaffected by ICS 205-930, were antagonized by methiothepin (0.1 μmol/l) and, even more potently, by ketanserin (1 μmol/l) (Figure 4). Figure 5 shows the effects of methiothepin (0.1 μmol/l), metergoline (0.1 and 1 μmol/l), ketanserin (1 μmol/l), cyanopindolol (1 μmol/l) and rauwolscine (1 μmol/l) on the responses to sumatriptan. The sumatriptan-induced contractions remained unaffected by ketanserin, but methiothepin, metergoline, cyanopindolol and rauwolscine behaved as antagonists (Figure 5; Table 3).

Table 3. Approximate pK_B values of various antagonists against the agonists indicated.

Antagonist	Concentration	n	Agonist	pK_B (±SEM) [a]
Ketanserin	1 μmol/l	5	5-HT	Not calculated [b]
	1 μmol/l	5	5-CT	Not calculated [b]
	1 μmol/l	3	α-Methyl-5-HT	7.1 (0.1)
	1 μmol/l	5	DOI	7.1 (0.3)
	1 μmol/l	5	2-Methyl-5-HT	Not calculated [c]
	1 μmol/l	8	Sumatriptan	No antagonism
Methiothepin	0.1 μmol/l	5*	5-HT	7.1 (0.4)
	0.1 μmol/l	6	5-CT	Not calculated [b]
	0.1 μmol/l	3	RU 24969	7.9 (0.8)
	0.1 μmol/l	5	2-Methyl-5-HT	7.3 (0.1)
	0.1 μmol/l	4	Sumatriptan	7.3 (0.1)
Methysergide	1 μmol/l	3	5-HT	Not calculated [c]
Rauwolscine	1 μmol/l	6	Sumatriptan	6.7 (0.2)
Metergoline	0.1 μmol/l	4	Sumatriptan	6.9 (0.3)
	1 μmol/l	4	Sumatriptan	7.3 (0.3)
Cyanopindolol	1 μmol/l	4	Sumatriptan	6.5 (0.3)
Flesinoxan	30 μmol/l	3	5-HT	5.3 (0.2)
ICS 205-930	1 μmol/l	3	5-HT	5.9 (0.1)
	1 μmol/l	5	2-Methyl-5-HT	No antagonism

[a], For calculation of pK_B only paired experiments were taken into account unless marked by *; [b], Not calculated due to biphasic nature of antagonism; [c], Not calculated because concentration-response curve did not reach a plateau; *n*, Number of observations.

4.4 Discussion

Previous studies [4-7, 13] in the human isolated saphenous vein have revealed that 5-HT-induced contractions were: (i) partially antagonized by spiperone and ketanserin, both of which preferentially affected the contractions following high concentrations of 5-HT; and (ii) mimicked by 5-CT, sumatriptan, RU 24969, α-methyl-5-HT, 5-methoxy-tryptamine and 8-OH-DPAT. Since contractions to α-methyl-5-HT and high concentrations

of 5-HT were antagonized by ketanserin and since the effect of sumatriptan was antagonized by methiothepin but not markedly by ketanserin, both 5-HT_1-like and 5-HT_2 receptors seem to be present in the human saphenous vein [6, 13].

The frame-work of 5-HT receptor classification, originally proposed by Bradley and colleagues [14], has now been expanded [15] and at least four types of 5-HT receptors (5-HT_1-like, 5-HT_2, 5-HT_3 and 5-HT_4) are recognized (see Ref. 16). It is well established that the 5-HT_1-like receptor category is heterogeneous in nature (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}) and even this subdivision seemingly does not completely correspond to the functional responses observed [16-18]. It is with this background that we have attempted to further characterize the human saphenous vein 5-HT receptors by using a large number of 5-HT receptor agonists and antagonists.

Identification of the 5-HT receptors

The presence of a contractile 5-HT_1-like receptor in the human saphenous vein is revealed by the fact that: (i) 5-CT, sumatriptan, RU 24969 and 8-OH-DPAT, all of which have more or less selective agonist action at 5-HT_1-like receptors, elicited saphenous vein contractions; (ii) the contractions elicited by sumatriptan were resistant to blockade by ketanserin, but were antagonized by methiothepin and other compounds with affinities for 5-HT_1 binding sites (metergoline, cyanopindolol and rauwolscine); and (iii) methiothepin clearly antagonized the responses to RU 24969. It should, however, be noted that the putative 5-HT_{1A} receptor agonist flesinoxan [19, 20] did not behave as an agonist, but rather as a weak antagonist of the responses to 5-HT; such an antagonist effect of flesinoxan has been described at the 5-HT_1-like receptor in the rabbit saphenous vein [21]. Lastly, despite the above evidence for the presence of 5-HT_1-like receptors in the saphenous vein, we have no adequate explanation why methiothepin, in contrast to its interaction with sumatriptan and RU 24969, did not cause a parallel shift of the lower part of the concentration-response curve of 5-CT. Possibly 5-CT acts as an agonist on other receptors than the 5-HT_1-like receptor present in this vessel; the antagonism by ketanserin of the upper part of the 5-CT-curve indicates that 5-CT activates 5-HT_2 receptors, especially at high concentrations. This phenomenon has been observed before in the human coronary artery [22] and the rabbit aorta [23].

Our experiments also provide evidence in favour of 5-HT_2 receptor-mediated contractions of the human saphenous vein. Thus, the contractile effects of 5-HT were antagonized by ketanserin in a clearly biphasic manner, mainly affecting the higher concentrations of 5-HT. Moreover, the relatively selective 5-HT_2 receptor agonist DOI, as well as α-methyl-5-HT, caused concentration-dependent saphenous vein contractions that

were also antagonized by ketanserin (Figure 4). The calculated pK_B values (7.1 against both 5-HT_2 receptor agonists) are admittedly somewhat low compared to those in other tissues [24, 25]. It is, however, possible that the blocking activity of ketanserin is being underestimated because α-methyl-5-HT has appreciable binding as well as functional affinity for 5-HT_1 receptor subtypes [25-28]. The presence of both 5-HT_1-like and 5-HT_2 receptors in the saphenous vein is further strengthened by the nature of the effects of methiothepin and methysergide; these mixed 5-HT_1-like and 5-HT_2 receptor antagonists shifted the concentration-response curve of 5-HT in a near parallel manner.

We would like to draw attention to three other findings in our present investigation. Firstly, as found earlier, particularly at 5-HT_1-like receptors [16, 29], methysergide had a partial agonist action weakly contracting the saphenous vein. Secondly, 2-methyl-5-HT contracted the human saphenous vein (Figure 4). Since this compound is generally considered selective for the 5-HT_3 receptor [14, 16], it may be argued that 5-HT_3 receptors also mediate contraction in this tissue. However, as also recently found in the rabbit isolated renal artery [30], the contractions elicited by 2-methyl-5-HT were not attenuated (perhaps somewhat potentiated) by ICS 205-930. The low pD_2 value of 2-methyl-5-HT (4.8±0.2) and the antagonism of its effects by methiothepin and, particularly, ketanserin strongly suggests that 2-methyl-5-HT contracted the human saphenous vein via 5-HT_2 receptors. However, in view of the tenfold higher affinity of 2-methyl-5-HT for the 5-HT_1 receptor subtypes than for the 5-HT_2 receptor [26, 28], an additional involvement of 5-HT_1-like receptors can not be excluded. Thirdly, in the absence of 5-HT_3 receptors, the ability of ICS 205-930 (1 μmol/l) to weakly attenuate 5-HT-induced contractions may point to the existence of 5-HT_4 receptors in the human saphenous vein. On the other hand, though present in the brain [15], heart [31-33] and gastro-intestinal system [34], the 5-HT_4 receptor has not yet been located in blood vessels.

Is the 5-HT_1-like receptor related to 5-HT_1 binding site subtypes?

Since receptor binding experiments clearly distinguish at least four 5-HT_1 binding site subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D}) (see Ref. 26, 35, and 36), we compared agonist pD_2 and antagonist pK_B values obtained in the present experiments with the binding affinities at the above 5-HT_1 binding subtypes (Figure 6). Admittedly, such a comparison is slightly hampered by the fact that we deal with two different receptor populations (5-HT_1-like and 5-HT_2) in the saphenous vein. In order to counter this potential disadvantage, we chose agonists (including 5-HT) with a relatively high affinity for 5-HT_1-like receptors and the pK_B values of antagonists against the selective 5-HT_1-like receptor agonist sumatriptan. The above comparison yielded the following correlation

coefficients (r): 0.12, 0.74, 0.40, 0.97 and 0.98 for the agonists; and 0.65, 0.45, 0.21, 0.77 and 0.89 for the antagonists at, respectively, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D} (calf caudate) and 5-HT_{1D} (human caudate) receptors. The correlation at the 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1C} was not only lower than that at the 5-HT_{1D} receptors (both in the human and calf caudate membranes), but also a theoretical straight line connecting the correlation points (see Figure 6) for these three 5-HT_1 binding subtypes (but not 5-HT_{1D}) could be rejected (α=0.05). Therefore, the 5-HT_1-like receptor mediating contraction of the human isolated saphenous vein resembles the 5-HT_{1D} receptor subtype, a conclusion that is also supported by data reported earlier [7, 37]. However, this receptor may not be entirely identical to the 5-HT_{1D} receptor identified in the calf or human caudate membranes, since the order of binding affinity at the 5-HT_{1D} receptor site of methiothepin and metergoline is contradictory to the potency order of functional antagonism against sumatriptan. An analogue observation was made by Hamel and Bouchard [38] for the 5-HT_{1D} receptor contracting the human isolated pial arteries. Indeed, Sumner and Humphrey [39] have already proposed a subdivision for 5-HT_{1D} receptors, and the primary structure and function of the cloned 5-HT_{1D} receptor [40] shows several marked discrepancies with the published 5-HT_{1D} receptor affinities [35].

Comparison between ungrafted and grafted saphenous veins

Apart from a recent report on a 12-year old saphenous vein graft [41], we are not aware of any other pharmacologic investigations in such vessels. As also observed by us in the microscopic sections (Figure 3), Steen and colleagues found a marked proliferation of intimal and smooth muscle layers [41]. Their pharmacological findings indicated that α-adrenoceptors mediating contraction of this grafted venous segment were 'atypical' in nature. However, with regard to 5-HT receptors, we observed no obvious change in the three grafted vessel segments studied by us; the pD_2 values as well as the maximal effects of 5-HT and sumatriptan, expressed as a percentage of potassium-induced contractions, were not different from those in the ungrafted saphenous veins. But, it appears that the general ability to contract, as indicated by the contractions to potassium (Table 2), was substantially less in the grafted vessels. Possibly, the mechanical inhibition by newly formed intimal myofibroblasts, and altered organization of areas of the medial smooth muscle layers, lead to an overall less effective contractile response. By contrast, in venous grafts in animals, others have reported hyper-reactivity to 5-HT, which correlated with intimal thickening [42, 43]. However, we only had the opportunity to study grafted saphenous veins from three patients undergoing heart transplantation who are likely to have more extensive pathological lesions than those individuals undergoing coronary bypass surgery.

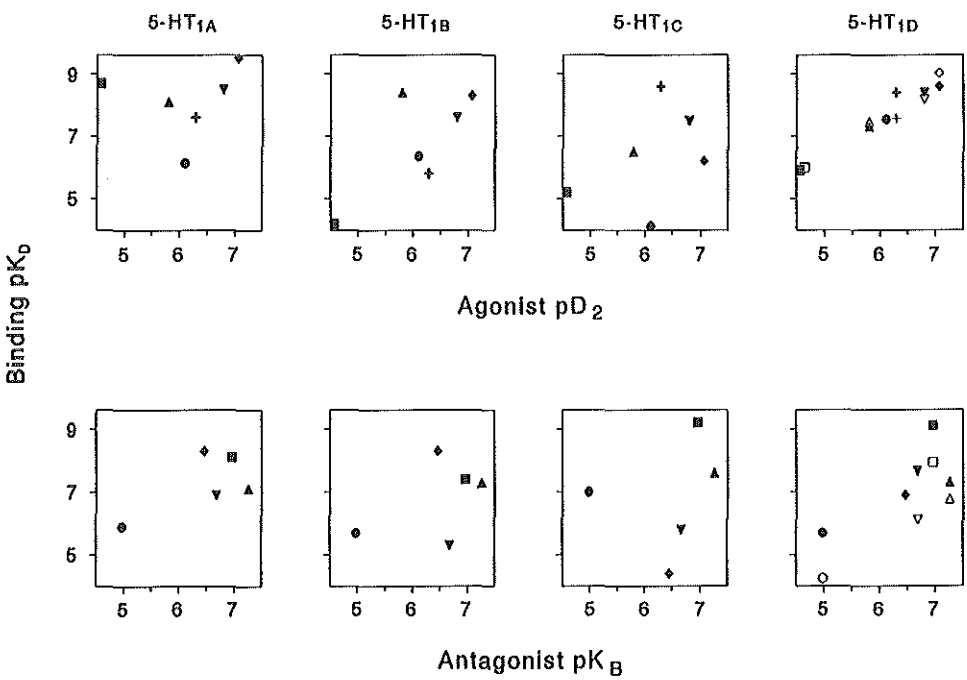

Figure 6. Correlation between agonist pD_2 or antagonist pK_B (against sumatriptan) values obtained in our functional tests (Table 1 and 3) and pK_D (expressed as -Log mol/l) values for 5-HT_1 binding site subtypes. All binding data are from Ref. 26, except those for sumatriptan [36] and for 5-HT_{1D} binding in the human caudate membrane [35]. Receptor binding was studied by the quoted authors in the following tissues: 5-HT_{1A}, pig frontal cortex; 5-HT_{1B}, rat frontal cortex; 5-HT_{1C}, pig choroid plexus; and 5-HT_{1D}, calf (filled symbols) or human (open symbols) caudate membranes. For ketanserin a pK_B value of 5.0 was calculated from four paired experiments. For metergoline a pA_2 of 7.0 was calculated using multiple regression analysis. Symbols for agonists: ▼, 5-HT; ◆, 5-CT; +, methysergide; ●, sumatriptan; ▲, RU 24969 and ■, 8-OH-DPAT. Symbols for antagonists: ■, metergoline; ▲, methiothepin; ▼, rauwolscine; ◆, cyanopindolol; ●, ketanserin. For correlation coefficients, see text.

Comparison with 5-HT receptors in other human vessels

Extensive studies characterizing 5-HT receptors in the human basilar [44] and pial [38] arteries have been performed. Some important differences between the results obtained here and those reported earlier may be pointed out. Firstly, in the basilar and pial arteries 5-HT-induced contractions were unaffected by ketanserin, indicating the absence of 5-HT_2 receptors. Secondly, unlike our experiments, 2-methyl-5-HT was ineffective in the basilar artery; however, despite the fact that contractile 5-HT_3 and 5-HT_2 receptors are absent, pial arteries did contract to 2-methyl-5-HT, presumably via a 5-HT_1-like mechanism. Thirdly, (±)cyanopindolol did not affect sumatriptan-induced contractions in the basilar artery, but the concentrations used were ten times lower (0.1 μmol/l) than in our experiments. Lastly, the affinity for methiothepin was higher in the basilar (pK_B: 8.8) or pial (pA_2: 8.55) arteries than in our experiments (pK_B: approximately 7.1). It is possible that the 5-HT_1-like receptor subtype present in the saphenous vein may not be identical with that present in the basilar arteries. However, as in the present experiments, a high correlation was found between the functional pD_2 values in the pial arteries and the pK_D values for the 5-HT_{1D} binding sites in the human caudate membranes [38].

Like in the saphenous vein, both 5-HT_1-like and 5-HT_2 receptors have been reported in the human coronary artery and similar pD_2 values for sumatriptan, 5-HT and 5-CT have been found [22, 44, 45]. However, at this time not enough experimental data in human isolated coronary artery is available to make a valid comparison between the 5-HT_1-like receptor subtype contracting coronary artery and saphenous vein.

In conclusion, a mixed population of 5-HT_1-like and 5-HT_2 receptors is present in the human isolated saphenous vein. Low concentrations of 5-HT contract the vessel mainly via the 5-HT_1-like receptor, but at higher concentrations 5-HT_2 receptors are recruited as well. This 5-HT_1-like receptor in the saphenous vein closely resembles the 5-HT_{1D} binding site. These results imply that, should 5-HT be involved in the pathophysiology, 5-HT_2 receptor antagonists, such as ketanserin, would not be entirely effective in preventing peri- or postoperative spasm of the saphenous vein graft. Since the human coronary vessels also contain 5-HT_1-like and 5-HT_2 receptors, the use of a mixed 5-HT_1-like / 5-HT_2 receptor antagonist may well be more efficacious in ameliorating coronary artery or saphenous vein graft spasm.

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Chapter 5

5-Hydroxytryptamine receptors mediating contractions of the human isolated coronary artery*

Summary - We investigated contractile responses of the human isolated coronary artery to 5-HT, human washed platelets, sumatriptan and ergotamine. 5-HT (pD_2: 6.8 ± 0.1, E_{MAX}: 47.7 ± 6.8 mN) and platelets (effect 14.4 ± 2.8 mN with 3.10^{10} platelets/l) caused contractile responses which were attenuated by ketanserin (1 μM). In the presence of ketanserin (1 μM), both rauwolscine (1 and 10 μM) and cyanopindolol (1 and 10 μM) caused concentration-dependent additional antagonism against leftover contractions induced by low (≤ 1 μM) concentrations of 5-HT. Sumatriptan-induced contractions (pD_2: 6.2 ± 0.1; E_{MAX}: 10.7 ± 2.4 mN) were antagonized to a similar extent by both rauwolscine (1 μM) and cyanopindolol (1 μM) (pK_B: 6.5 ± 0.1 and 6.4 ± 0.1, respectively) and also by metergoline (0.1 μM; pK_B:7.2 ± 0.1). The order of potency of antagonists against sumatriptan resembles the order reported for the human saphenous vein 5-HT_{1D}-like receptor. Against platelet-induced contractile responses we observed no significant additional antagonism by cyanopindolol (1 μM) or rauwolscine (1 μM). Ergotamine caused potent contractile responses (pD_2: 8.4 ± 0.3, E_{MAX}: 19.4 ± 2.4 mN). It is concluded that, although 5-HT_2 receptors predominantly mediate 5-HT-induced contractions, the 5-HT_1-like receptor seems to play a role in coronary vasospasm caused by low concentrations of 5-HT.

*, *Based on:* Bax, W.A., Renzenbrink, G.J., Van Heuven-Nolsen, D., Thijssen, H.J.M., Bos, E. and Saxena, P.R. (1993) 5-HT receptors mediating contractions of the isolated human coronary artery. *Eur. J. Pharmacol.* **239**, 203-210.

5.1 Introduction

5-Hydroxytryptamine (5-HT) released from aggregating platelets is thought to play a role in the cascade of events that leads to an acute ischemic state of the heart such as in unstable angina or Prinzmetal's angina pectoris [1-3]. Indeed, 5-HT is well known to contract the human coronary artery both *in vitro* [4-7] and *in vivo*; the latter only in case of high concentrations of 5-HT [8], or in case of a diseased coronary artery [9, 10]. The healthy coronary artery was found to dilate after infusion of low concentrations of 5-HT *in vivo*, and this dilatation was potentiated by ketanserin, a 5-HT_2 receptor antagonist [9]. The human isolated coronary artery contracts to 5-HT via both 5-HT_1-like and 5-HT_2 receptors. Predominant mediation by the 5-HT_2 receptor [4, 5] but also by a 5-HT_1-like receptor [6] has been suggested. Despite involvement of 5-HT_2 receptors, ketanserin has been shown to be clinically ineffective against vasospastic angina [11] or ergometrine-induced coronary artery constriction [12]. This has tempted investigators to speculate on the potential beneficial effects of a mixed 5-HT_1-like / 5-HT_2 receptor antagonist over a selective 5-HT_2 receptor antagonist [8, 13-15]. Interestingly, the receptor binding affinity of 5-HT for the 5-HT_1-like receptor subtypes is more than 100-fold higher than that for the 5-HT_2 receptor [16]. Indeed, in the human saphenous vein, which also contracts to 5-HT via a mixed 5-HT_1-like / 5-HT_2 receptor population, low concentrations of 5-HT were found to mediate contractions mainly via a 5-HT_1-like receptor [15, 17]. In the human coronary artery, this may be of particular interest since studies of this vessel have revealed a ketanserin-resistant [6, 8] or 5-HT_1-like contractile receptor [7], possibly related to the presence of atherosclerotic plaques.

In the present study we have therefore investigated the effect of two 5-HT_1-like receptor antagonists (cyanopindolol and rauwolscine) in the presence of the 5-HT_2 receptor blockade by ketanserin on contractions induced by 5-HT and human activated platelets. Rauwolscine and cyanopindolol have high affinity for α_2 and ß adrenoceptors respectively, but they also have affinity for 5-HT_{1D} and 5-HT_{1A} receptor subtypes, especially in comparison to 5-HT_2 receptors [16]. Using these two antagonists and also metergoline, we further investigated the nature of the 5-HT_1-like receptor involved.

Also the recent development of a novel anti-migraine drug, sumatriptan, a selective 5-HT_1-like receptor agonist [18], has raised interest in the relevance of contractile 5-HT_1-like receptors and possible coronary side-effects [19, 20]. We therefore compared the contractile effect of sumatriptan to that of ergotamine, an anti-migraine drug with well documented coronary side-effects (see Ref. 21) and in clinical use since many decades.

5.2 Materials and methods

Tissue preparation

The right epicardial coronary artery was obtained from 21 heart beating organ donors who died of non-cardiac disorders less than 24 h before the tissue was taken to the laboratory (8 cerebrovascular accident, 8 polytrauma, 4 cerebral hypoxia, 1 pulmonary embolus; 14 male, 7 female; age 1-49 years). The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services Foundation / Eurotransplant Foundation) after removal of the aortic and pulmonary valves for homograft valve transplantation. The study was approved by the Ethical Committee of the University Hospital Rotterdam 'Dijkzigt'. The hearts were stored at 0-4 °C in a sterile organ protecting solution (UW, EuroCollins, or HTK-Brettschneider; see Ref. 22) immediately following circulatory arrest. After arrival in the laboratory, the right coronary artery was removed and placed in a cold, oxygenated Krebs bicarbonate solution of the following composition: 118 mM sodium chloride, 4.7 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium sulphate, 1.2 mM potassium dihydrogenphosphate, 25 mM sodium bicarbonate and 8.3 mM glucose; pH 7.4. The vessel was cut into rings of approximately 4 mm of length and suspended on stainless steel hooks in 8 ml organ baths containing the Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37° C. Vessel segments containing macroscopically visible atherosclerotic lesions, were not used in the present study. The segments were allowed to equilibrate for at least 30 min and washed every 15 min. Changes in tension were recorded using a Harvard isometric transducer. Preparations were stretched to a pretension of 20 mN. The tissue was exposed to K^+ (30 mM) twice. Subsequently, the functional integrity of the endothelium was verified by observing relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). Five segments from two hearts did not relax to substance P, and were discarded from the experiments. After washout, the tissue was exposed to 100 mM K^+ to determine the maximal contractile response to K^+. The tissue was then allowed to equilibrate in the Krebs solution for a period of 30 min.

Determination of agonist and antagonist potency

After this period of equilibration, some segments remained untreated (controls), whereas others were incubated with a receptor antagonist for thirty minutes. After that, a cumulative concentration response curve was obtained on every segment until the maximum response (E_{MAX}) was reached. Responses were expressed as increase of tension

(mN). Curves obtained in the presence of a receptor antagonist were compared to the control curve. All curves were obtained in a paired, parallel experimental set-up.

Isolation of platelets and platelet experiments

Approximately 23 ml of blood was obtained from each of 6 healthy male donors, age 26-52 years, who had not taken anti-platelet drugs for at least 14 days prior to the experiment. Platelets were isolated essentially according to Ref. 23. In brief, the blood was centrifuged for 40 min at 55 g and 20 °C, after which the platelet rich plasma was pipetted off and an equal volume of citrate anticoagulant solution (5 mM potassium chloride, 105 mM glucose, 93 mM sodium citrate, 7 mM citric acid; pH 6.5) was added. Centrifugation for 20 min at 570 g (20 °C) produced a platelet pellet which was resuspended in 2.5 ml of the citrate solution. The platelet concentration in the obtained suspension was determined using a Platelet Analyzer, Hematology Series 810 (Baker Instruments, Allentown PA, USA). The platelets were added in a cumulative manner to the organ baths in volumes to result in concentrations of 10^9 - 3.10^{10} platelets per litre. Responses were expressed as increase of tension (mN). Care was taken to minimize the amount of time during which the platelets had to be kept before adding (less than 35 min at room temperature). A sample (500 µl), taken thirty min after the last platelet concentration had been added, was centrifuged for 20 min at 570 g in the presence of 30 µM indomethacin, pipetted off and frozen at -70 °C until 5-HT was determined using high performance liquid chromatography (HPLC) with electrochemical detection.

Analysis of Data

Curves that covered the full sigmoidal range were analyzed by means of a computerized curve fitting technique [24] to obtain E_{MAX} (maximal response) and pD_2 (-Log of the molar concentration of an agonist needed to reach half of its maximal effect, i.e. $-LogEC_{50}$) values, which were averaged for the respective agonists. For antagonists against sumatriptan, approximate pK_B values were calculated, assuming the nature of antagonism to be competitive, by the equation described by Furchgott [25]:

$$pK_B = -Log[B] + Log\{([A_2]/[A_1])-1\},$$

where [B] is the antagonist concentration and $[A_1]$ and $[A_2]$ are, respectively, the EC_{50} values of the agonist in the absence and presence of [B]. For calculation of approximate pK_B values only paired experiments were taken into account.

All data are presented as mean ± S.E.M.. Multiple analysis of variance (MANOVA) followed by Student's *t*-test for paired data was used for comparison of mean contractile

responses. pD_2 and E_{MAX} of sumatriptan-induced contractions without or in the presence of an antagonist were compared using a paired Student's t-test. A correlation coefficient was calculated according to Pearson. A P value less than 0.05 was assumed to denote a significant difference.

Compounds

The following drugs were used in this study: cyanopindolol (gift: Sandoz AG, Basel, Switzerland), ergotamine tartrate (Pharmacy A.Z.R.-Dijkzigt, Rotterdam, the Netherlands), 5-hydroxytryptamine creatinine sulphate (5-HT; Sigma Chemical Co., St. Louis, USA), ketanserin tartrate (gift: Janssen Pharmaceutica, Beerse, Belgium), metergoline (gift: Farmitalia, Milan, Italy), prostaglandin $F_{2\alpha}$ (tris salt), substance P acetate (both: Sigma Chemical Co., St. Louis, USA), rauwolscine hydrochloride (Fluka AG, Buchs, Switzerland) and sumatriptan (gift: Glaxo, Ware, U.K.).

5.3 Results

The effect of substance P and potassium

Vessel segments relaxed to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ (1 μM) with 75.9 ± 6.1% (range 15-108%) of the contractile response to prostaglandin $F_{2\alpha}$, which is in accordance with previous studies on substance P in the human isolated coronary artery obtained from patients undergoing cardiac transplantation [26-28]. K^+ (100 mM) caused a mean contractile response of 47.3 ± 3.5 mN. No difference in the mean contractile response to K^+ (100 mM) was observed in any of the compared groups.

Analysis of the responses to 5-HT, sumatriptan and human washed platelets

5-HT caused concentration dependent contractions of the vessel segments (pD_2: 6.8 ± 0.1, E_{MAX}: 47.7 ± 6.8 mN, n=11). Ketanserin (1 μM) biphasically antagonized 5-HT-induced contractions (n=11). The 5-HT_1-like receptor antagonists cyanopindolol and rauwolscine (both: 1 μM, n=11 and 10 μM, n=6) significantly attenuated the leftover first phase in the presence of ketanserin (i.e. 5-HT concentration ≤ 1 μM) in a concentration-dependent manner (Figure 1).

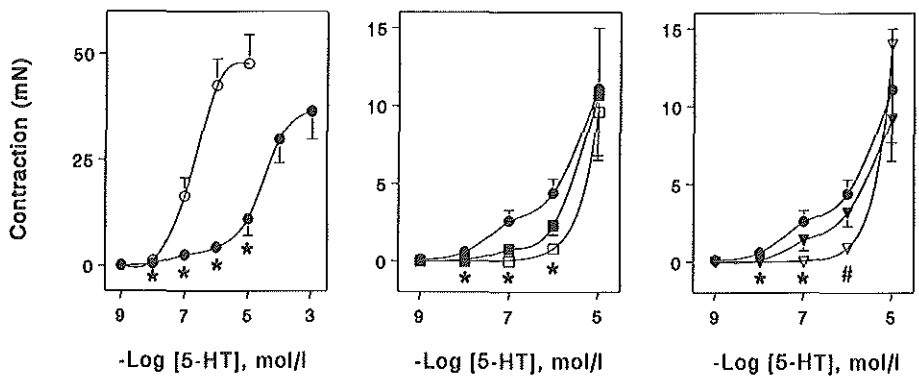

Figure 1. Contractions of the human isolated coronary artery to 5-HT. Control concentration response curves (n=11, ○) or curves made in the presence of ketanserin (1 μM, n=11, ●), ketanserin (1 μM) combined with rauwolscine (1 μM, n=11, ▩; 10 μM, n=6, □) or ketanserin (1 μM) combined with cyanopindolol (1 μM, n=11, ▾; 10 μM, n=11, ▿). Contractions are expressed in mN increase of tension. *, Left panel: statistically different ($P<0.05$) from the control response; *, Middle and right panel: both 1 μM and 10 μM of rauwolscine and cyanopindolol cause significant ($P<0.05$) attenuation compared to contraction in the presence of ketanserin (1 μM) alone; #: Only 10 μM cyanopindolol causes significant ($P<0.05$) additional antagonism, when compared to curve in the presence of ketanserin (1 μM) alone.

Sumatriptan generated concentration-dependent coronary artery contractions (n=8; pD_2: 6.2±0.1; E_{MAX}:10.7 ± 2.4 mN or 22% of the maximal response to 5-HT). A significant rightward shift of the sumatriptan-induced concentration response curve was caused by rauwolscine (1 μM) or cyanopindolol (1 μM) and also metergoline (0.1 μM) (Figure 2). A pK_B value of 6.5 ± 0.1, 6.4 ± 0.1 and 7.2 ± 0.1 respectively, was calculated for these antagonists.

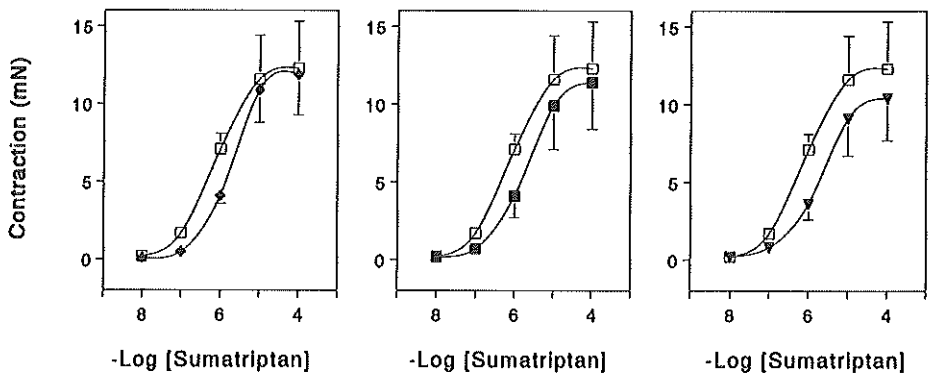

Figure 2. Contractions of the human isolated coronary artery to sumatriptan (n=5) without (□, control) or in the presence of metergoline (0.1 μM, ♦), rauwolscine (1 μM, ■) or cyanopindolol (1 μM, ▾). The concentration of sumatriptan refers to mol/l. Contractions are expressed in mN increase of tension.

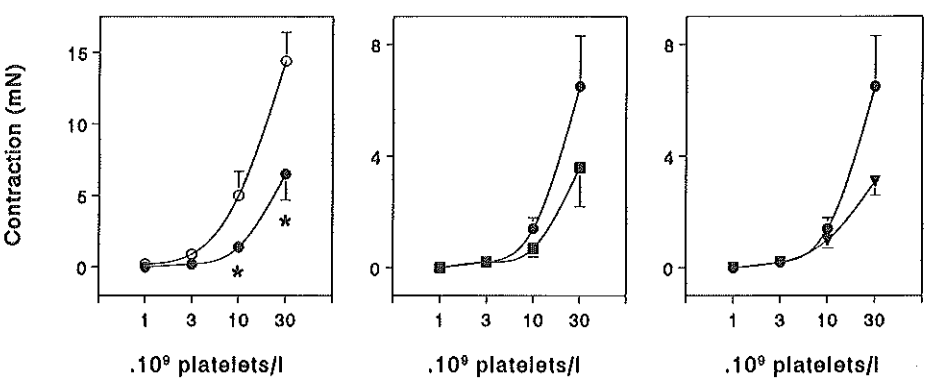

Figure 3. Contractions of the human isolated coronary artery to human platelets (n=6). Control concentration response curves (○) or curves made in the presence of ketanserin (1 μM, ●), ketanserin combined with rauwolscine (both 1 μM, ■) or ketanserin combined with cyanopindolol (both 1 μM, ▾). Contractions are expressed in mN increase of tension. *, statistically different ($P<0.05$) from control response.

Human washed platelet-induced contractions reached 14.4 ± 2.8 mN. At the highest platelet concentration tested, the concentration response curve had not reached the maximum response (Figure 3). Since one is restricted with the amount of blood that can be drawn from the platelet donor, higher platelet concentrations were not used. Ketanserin (1 μM) antagonized contractions induced by platelets to reach 6.5 ± 1.8 mN. Against leftover platelet-induced contractions in the presence of ketanserin (1 μM), rauwolscine (1 μM) or cyanopindolol (1 μM) did not cause significant additional antagonism of the contractile response (Figure 3). The concentration of 5-HT, measured in the organ bath 30 min after adding the highest platelet concentration, was 65 ± 10 nM.

The pD_2 and E_{MAX} values for sumatriptan and 5-HT, or the maximal effect of platelets, did not correlate with the functional endothelial integrity, as determined by relaxation to substance P ($0.03 \leq r \leq 0.64$; $p \geq 0.12$).

The effect of ergotamine

Ergotamine caused a concentration dependent contractile response of the vessel segment, reaching 19.4 ± 2.4 mN or 40.7% of 5-HT-induced contractions (n=6). The pD_2 of ergotamine was 8.4 ± 0.3 (Figure 4). A full concentration response curve to ergotamine took 54 ± 14 min, whereas a full concentration response curve to sumatriptan took 17 ± 1 min (data from six paired experiments).

5.4 Discussion

In the present study, we compared the effect of selective 5-HT_2 receptor antagonism to the effect of mixed 5-HT_2/5-HT_1-like receptor antagonism on contractions evoked both by 5-HT and activated human washed platelets. Moreover, we attempted to further characterize the 5-HT_1-like receptor subtype, using the selective 5-HT_1-like receptor agonist sumatriptan as a tool. We showed that ketanserin significantly attenuated 5-HT- and/or platelet-induced contractions. For 5-HT, this inhibition was biphasic. In the presence of ketanserin, rauwolscine and cyanopindolol caused a significant, concentration dependent further attenuation of contractions induced by low (≤ 1 μM) concentrations of 5-HT. Sumatriptan-induced contractions were competitively antagonized to a similar extent by both rauwolscine (1 μM) and cyanopindolol (1 μM) and also by metergoline (0.1 μM). In platelet experiments, cyanopindolol and rauwolscine did not contribute significantly to

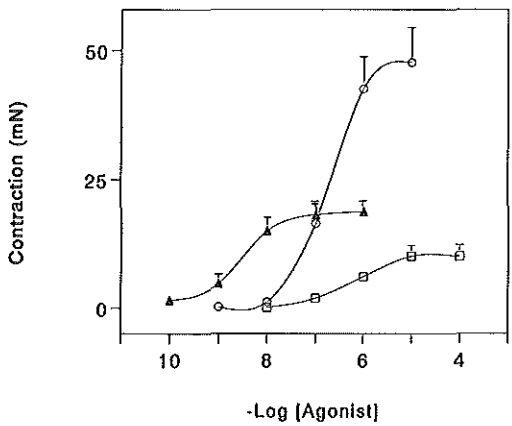

Figure 4. Contractions of the human isolated coronary artery to the anti-migraine drugs ergotamine (▲) and sumatriptan (□), and 5-HT (○); n=6-11. Agonist concentration refers to mol/l. Contractions are expressed in mN increase of tension.

antagonism produced by a combination of 5-HT_1-like and 5-HT_2 receptor antagonists (Figure 3). There may, however, be a trend similar to data obtained with 5-HT (Figure 1). The present results are to a certain extent in keeping with those of other investigators [4, 5], who concluded that, in terms of efficacy, human coronary artery contractions to 5-HT are mediated predominantly via 5-HT_2 receptors. These authors drew their conclusions from the fact that ketanserin was a potent antagonist of 5-HT-induced contractions and that contractions to sumatriptan of the human coronary artery amounted only up to 21% of the maximal response to 5-HT [4]. Later, Chester and colleagues [7] showed that methiothepin antagonized sumatriptan-induced contractions, whereas ketanserin and MDL 72222 (a 5-HT_3 receptor antagonist) did not have such an effect, indicating that sumatriptan contracts the human coronary artery via a population of contractile 5-HT_1-like receptors.

The significance of contractile 5-HT_1-like receptors as compared to 5-HT_2 receptors

From a theoretical point of view one would expect that, given the presence of a mixed receptor population mediating contraction, low concentrations of 5-HT activate primarily the 5-HT_1-like receptor since 5-HT has approximately 100-fold higher affinity for 5-HT_1-receptor subtypes than for 5-HT_2 receptor subtypes in the brain [16]. On the other

hand, in view of functional pD_2 values in 5-HT_1-like and 5-HT_2-receptor assays (e.g. dog saphenous vein and rabbit aorta [29]), one could argue that the difference in affinity for *vascular* receptors may be less, or that this difference is blunted by second messenger mechanisms. When considering the potential advantages of a mixed 5-HT_2 / 5-HT_1-like receptor antagonist, a possible difference in receptor affinity suggests that, at low concentrations of 5-HT, the *relative* contribution of the 5-HT_1-like receptor antagonism would be increased when compared to the contribution of 5-HT_2 receptor antagonism. In our experiments, ketanserin (1 μM) reduced the small contractile response elicited by 0.01 μM 5-HT from 1.2 ± 0.3 mN to 0.6 ± 0.1 mN. Both cyanopindolol and rauwolscine (10 μM) completely and significantly abolished the small leftover contractile response. Although indeed the contractions caused by this concentration of 5-HT are small, these data indicate that, at this concentration of 5-HT, approximately 50% of the contractile response was mediated by 5-HT_1-like receptors. At 5-HT concentrations of 0.1 μM and 1 μM the relative contribution of the 5-HT_1-like receptor antagonism to total antagonism (i.e. by the combinations of ketanserin and rauwolscine or cyanopindolol) decreased to approximately 16% and 9%, respectively.

At present, to our knowledge, one can only speculate on the actual concentration of 5-HT *in vivo*, when platelets aggregate close to the vascular smooth muscle cell. The concentration of 5-HT, measured in the organ bath after addition of 3.10^{10} platelets per litre, was 65 ± 10 nM. The concentration of 5-HT, as measured by Van den Berg and co-workers [2] in the aorta *in vivo* was approximately 85 nM, which may relate to 5-HT concentrations near the smooth muscle cell, but which could also be much lower. In this respect, it is important to keep in mind that the platelet-release of 5-HT is accompanied by several other contractile (thromboxane A_2, TxA_2, and prostaglandin E_2, PGE_2) but also relaxant (adenosine diphosphate, ADP) agents [1]. The endothelium-dependent relaxant effect by the latter, however, appears to be of less significance in atherosclerotic patients. In cooperation with the cyclooxygenase-derived contractile agonists, a low concentration of 5-HT may cause severe vasoconstriction. Therefore, it is of particular interest that several authors have suggested synergistic interaction between 5-HT- and thromboxane A_2-receptor-mediated responses [30-33]. Interestingly, in the rabbit femoral artery, which also contains contractile 5-HT_1-like and 5-HT_2 receptors, the presence of the stable thromboxane A_2-mimetic U46619 altered the efficiency of 5-HT_1-like- but not 5-HT_2 receptor occupancy-effect coupling, thereby increasing the maximal effect to sumatriptan but not to α-methyl-5-HT [34].

Does the contractile 5-HT$_1$-like receptor resemble any of the known receptor subtypes?

We concentrated on the contractile response elicited by the selective 5-HT$_1$-like receptor agonist sumatriptan, as was also done previously in the human saphenous vein [15]. Metergoline (0.1 μM), rauwolscine (1 μM) and cyanopindolol (1 μM) all caused a small but significant rightward shift of the sumatriptan-induced concentration response curve. The order of potency of these antagonists (metergoline > rauwolscine ≈ cyanopindolol) was similar to the order found for antagonism against sumatriptan in the human saphenous vein, in which the receptor was characterized as being 5-HT$_{1D}$-like (human isolated saphenous vein: pK$_B$: 6.9 ± 0.3, 6.7 ± 0.2 and 6.5 ± 0.3, respectively; pD$_2$ sumatriptan: 6.1 ± 0.1, see Ref. 15). In this aspect, a shift of 1.05 log units, as found for methiothepin (0.1 μM) against sumatriptan-induced contractions of the human coronary artery [7], appears to be in line with the present results and the human saphenous vein 5-HT$_{1D}$-like receptor. An almost equal potency of rauwolscine and cyanopindolol, and the fact that ketanserin was inactive as an antagonist against sumatriptan [7] virtually rules out involvement of a contractile 5-HT$_{1A/1B}$ or a 5-HT$_{1C}$ (now: 5-HT$_{2C}$) receptor, respectively. Further characterization of the contractile coronary artery 5-HT$_1$-like receptor as one of the recently cloned 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptors [35] may benefit from mRNA identifying techniques more than from functional tests such as the organ bath. A remarkably enduring lack of sufficiently selective agonists and antagonists and a steadily increasing amount of closely related newly cloned receptor subtypes severely impairs detailed functional receptor analysis.

Contractile 5-HT$_1$-like receptors and atherosclerotic changes of the vascular wall

Contractile 5-HT$_1$-like receptors in the human coronary artery have been emphasized before [6], especially with respect to an increase in 5-HT$_1$-like receptor mediated responses in or near atherosclerotic areas of the vessel [7]. We have found no correlation between pD$_2$ and E$_{MAX}$ of sumatriptan, 5-HT or platelets and the relaxation to substance P after precontraction with prostaglandin F$_{2\alpha}$. Substance P relaxes the vessel via an endothelium-dependent (nitric oxide-mediated) mechanism, the effect of which is decreased in case of atherosclerosis [27-28]. In contrast to Chester and colleagues [7], who compared atherosclerotic segments to non-atherosclerotic segments within one patient, we have only studied macroscopically non-atherosclerotic tissue in which relaxation to substance P varied from 15 to 108% of precontraction. The latter variation may be a reflection of generalized intimal hyperplasia and early atherosclerosis (which were observed using light microscopy), whereas the differences found by Chester and co-workers [7] reflect localized atherosclerotic plaque formation. This discrepancy may be an explanation for the

divergence. Our data suggest, however, that there may not be an unequivocal link between atherosclerotic changes and the response to 5-HT related ligands. Similar conclusions were reached for the potency of 5-HT by Kaumann and Brown [6].

Comparison of the contractile effect of sumatriptan and ergotamine.

The potential coronary side-effects of the new anti-migraine drug sumatriptan have gained widespread attention [19, 20, 37]. First, it must be admitted that the concentration response curves of sumatriptan and ergotamine are not easily compared due to the extremely slow nature of the contractile response caused by ergotamine [38]. For this reason we have not further analyzed the receptor that mediates the contractile response to ergotamine. The slow response to ergotamine may have resulted in a somewhat underestimation of the ergotamine-induced contractile responses. Yet, ergotamine was functionally approximately 100-fold more potent than sumatriptan. Since the affinity of ergotamine and sumatriptan for the 5-HT_{1D} receptor is approximately similar [16], the major difference in functionally determined pD_2 values may well be due to the high affinity of ergotamine for the 5-HT_2 receptor (100 times that of 5-HT) [16, 39]. Sumatriptan, on the other hand, is selective for the 5-HT_1-like receptor [18]. Also the higher efficacy of ergotamine may indicate that other receptors than the 5-HT_1-like receptor are involved [40]. Indeed, it is not unlikely that α-adrenoceptors, dopamine- and other (as yet unknown) receptors are involved in the contractile response to ergotamine. Ergotamine has substantial affinity for e.g. α-adrenergic and dopamine receptors [41]. Our results suggest that the cardiac liability of ergotamine is higher than that of sumatriptan. Still, extreme care has to be taken when prescribing sumatriptan to possibly coronary artery compromised patients.

We conclude that the 5-HT_1-like receptor plays a role in coronary artery contractions induced by low concentrations of 5-HT (≤ 1 μM), although 5-HT_2 receptors predominantly mediate 5-HT-induced contractions [4, 5]. The contractile 5-HT_1-like receptor resembles the human saphenous vein 5-HT_{1D}-like receptor. Furthermore, ergotamine is 100-fold more potent than sumatriptan as a constrictor of the human isolated coronary artery.

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Chapter 6

Low dose aspirin inhibits platelet-induced contraction of the human isolated coronary artery; A role for additional 5-HT receptor antagonism against coronary vasospasm?*

Summary - *Background.* The beneficial effect of low dose aspirin in the prevention of coronary vasospasm is well documented. In this study we investigated the contractile effect of human washed platelets on the human isolated coronary artery. We concentrated on the effect of low dose aspirin (40 mg per day), taken by the platelet-donor and on the efficacy of thromboxane A_2 (TxA_2)- and 5-hydroxy-tryptamine (5-HT) receptor antagonists.

Methods and Results. Human coronary artery segments were suspended in an organ bath set-up for isometric tension measurement. Platelets (10^9-3.10^{10}/l) elicited concentration dependent contractile responses of the coronary artery segments reaching 28.4 ± 7.1% of contractions induced by 100 mM K^+. The contractile response tended to be decreased in vessel segments with histological signs of early atherosclerosis. Contraction was significantly attenuated after pretreatment of the vessel segments with ketanserin (5-HT_2 receptor antagonist, 1 μM) or SQ30741 (TxA_2 receptor antagonist, 0.01 μM), reaching 8.8 ± 2.3% and 3.2 ± 2.2% of contraction to 100 mM K^+, respectively. Platelets obtained from the same platelet-donors after taking aspirin (40 mg/day for 7-13 days) caused significantly lower contractile responses (7.6 ± 2.7% of 100 mM K^+) associated with an almost selective inhibition of the synthesis of thromboxane measured in the organ bath solution (untreated platelets: 2.19 ± 0.43 nM; aspirin-treated platelets: 0.66 ± 0.05 nM). The amount of 5-HT secreted in the organ bath remained unaltered (65.17 ± 9.94 nM and 64.03 ± 8.98 nM, respectively). This explains why ketanserin significantly attenuated

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the residual contractile responses caused by platelets obtained from aspirin-treated subjects, whereas SQ30741 caused minor, non-significant additional attenuation. *Conclusion.* The results of the present study therefore suggest that additional antagonism of the contractile 5-HT receptors in the coronary artery may increase the efficacy of low dose aspirin *in vivo*.

6.1 Introduction

Aspirin has been shown extensively to be effective in the prevention of a number of cardiovascular diseases. A daily dose of 325 mg reduced the risk of myocardial infarction by 44% [1]. A much lower dose of 20 to 40 mg already caused complete inhibition of the synthesis of thromboxane A_2 (TxA_2) [2], which, together with 5-hydroxytryptamine (5-HT, serotonin), are the major vasoconstrictor products involved in platelet-induced vasoconstriction [3, 4]. This same dose of aspirin (20-40 mg daily) hardly affected the endothelial production of prostacyclin (PGI_2), which has anti-aggregatory and vasorelaxant effects [5, 6]. Guided by the latter observations, clinical investigators have focused their attention on low doses of aspirin. Thus, 75 mg aspirin daily resulted in a significant (60%) reduction of the risk of myocardial infarction and subsequent death [7] in silent as well as in symptomatic ischemia [8]. An even lower dose of aspirin -30 mg- was found to be equally effective in the prevention of vascular events and had fewer adverse effects than a 283 mg dose in patients with a transient ischemic attack or minor stroke [9].

Damage of the endothelial lining of the vascular wall is thought to incite platelet adhesion to the exposed subendothelial collagen with the subsequent release of platelet products [10, 11]. Experimentally induced atherosclerosis in monkeys was found to alter the response to important platelet products like 5-HT, ADP and TxA_2 in a direction that would favour vasoconstriction when these products are released from aggregating platelets [12]. Indeed, elevated levels of both 5-HT [13] and thromboxane B_2 (TxB_2), the stable metabolite of TxA_2 [14, 15], have been observed in patients with coronary artery lesions and angina.

In the present experiments we investigated the effect of low dose aspirin, taken by the platelet-donors, on platelet-induced contractions of the human isolated coronary artery. We concentrated on the relative importance of cyclooxygenase products and 5-HT, before and after aspirin treatment. We attempted to relate the contractile responses to the organ bath concentration of various vasoactive compounds which are believed to be involved in

the generation and prevention of coronary vasospasm. Lastly, we microscopically examined sections of the vessel segments and tried to correlate early signs of atherosclerosis to functional responses.

6.2 Methods

Preparation of the tissue

Right epicardial coronary arteries were obtained from 10 heart beating organ-donors who had died of non-cardiac disorders less than 24 h before the tissue was taken to the laboratory (3 cerebrovascular accident, 3 polytrauma, 4 cerebral hypoxia; 8 male, 2 female; age 7-48 years). The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services / Eurotransplant Foundation) after removal of the aortic and pulmonary valves for valve transplantation. The study was approved by the Ethical Committee of the University Hospital Rotterdam 'Dijkzigt'. The hearts were stored at 0-4 °C in a sterile organ protecting solution immediately following circulatory arrest. After arrival in the laboratory, the right coronary artery was removed and placed in a cold, oxygenated Krebs bicarbonate solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KHPO_4$, 25 mM $NaHCO_3$ and 8.3 mM glucose; pH 7.4. Vessels were cut into rings of approximately 4 mm of length and suspended on stainless steel hooks in 8 ml organ baths containing the Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37° C. Vessel segments containing distinct, macroscopically visible atherosclerotic lesions, were not used.

Experimental protocol

The segments were allowed to equilibrate for at least 30 min and washed every 15 min. Changes in tension were recorded using a Harvard isometric transducer. Preparations were stretched to a pretension of 2 g. The tissue was exposed to potassium (30 mM) twice. Subsequently, the functional integrity of the endothelium was verified as relaxation to substance P (1 nM) after precontraction to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). One segment did not relax to substance P and was discarded from the experiments. After washout, the tissue was exposed to 100 mM potassium to determine the maximal contractile response. The tissue was then allowed to equilibrate for 30 min. A cumulative concentration response curve (CRC) was obtained after another 30 min period. During this period, some segments remained untreated (controls), whereas others were incubated in parallel with a

receptor antagonist. In some cases control curves were obtained in duplicate or triplicate, which were averaged and regarded as one curve in further analysis. The contractile response was expressed as a percentage of contraction induced by 100 mM potassium.

Isolation of platelets

Approximately 23 ml of blood was obtained from each of 6 healthy male donors (age: 26-52 yrs) who had not taken anti-platelet drugs for at least 14 days prior to the experiment. Platelets were isolated essentially according to Yang et al. (1991) [4]. In brief, blood was centrifuged for 40 min at 55 g and 20 °C, after which the platelet rich plasma was pipetted off and an equal volume of citrate anticoagulant solution (5 mM potassium chloride, 105 mM glucose, 93 mM sodium citrate, 7 mM citric acid; pH 6.5) was added. Centrifugation for 20 min at 570 g (20 °C) resulted in a platelet pellet which was resuspended in 2.5 ml of the citrate solution. The platelet concentration in the obtained suspension was determined using a Platelet Analyzer (Hematology Series 810, Baker Instruments, Allentown PA, USA). The suspension was added in a cumulative manner to the organ baths in appropriate volumes to result in bath concentrations of 10^9 - 3.10^{10} platelets/l. Since the platelets were readily activated after adding to the organ bath, resulting in platelet product release and consequential functional responses, we did not further regulate or induce the state of platelet activation. Care was taken to minimize the time during which the platelets had to be kept before adding (less than 35 min). After the first experiment the platelet-donors were treated with aspirin (acetyl salicylic acid, Pharmachemie b.v., Haarlem, The Netherlands); 40 mg/day, once daily at regular intervals) for a period until again human coronary tissue was available for experimentation. This period varied between 7 and 13 days. Then, another 23 ml of blood was drawn from the platelet-donor and the experiment was repeated, as described above.

Determination of the concentration of eicosanoids and 5-HT

Thirty minutes after adding the highest concentration of platelets, a sample of 1.8 ml was drawn from the organ bath in a polypropylene tube and indomethacin (30 μM) was added to stop cyclooxygenase activity. The samples were centrifuged at 570 g during 20 min. The solution above the pellet was pipetted off and stored at -80 °C until assay. The following eicosanoids were determined in this organ bath sample: TxB_2, PGE_2, and 6-keto $PGF_{1\alpha}$ (stable metabolite of PGI_2). Twenty microliter (TxB_2) and 100 μl (PGE_2 and 6-keto-$PGF_{1\alpha}$) portions of the organ bath solution were used for radioimmunoassay (RIA) [16]. Tritiated compounds were purchased from Amersham (U.K.), standards from Sigma Chem. Co. (St. Louis, Mo, USA) and antisera from Advanced Magnetics Inc.

(Cambridge, Ma, USA). The lower detection limits of the TxB_2, PGE_2, and 6-keto-$PGF_{1\alpha}$ assays were 1.25, 2.5, and 5 pg per tube, respectively. Radioactivity was determined by counting scintillation using a Packard 1500 Tricarb. Calculations were performed with additional software using spline functions.

5-HT was determined by high performance liquid chromatography (HPLC) with electrochemical detection. Organ bath solution (400 µl) was mixed with an equal amount of mobile phase and 20 µl samples were injected onto a reversed phase column (Bio-Sil C18 A/B, 5 µm, 150 x 4.6 mm, Bio-Rad Laboratories, Brussels, Belgium). The mobile phase consisted of 75 mM sodium acetate, 0.27 mM disodium EDTA, 2.13 mM heptane sulphonic acid and 20% methanol, pH 4.15 (flow rate: 0.8 ml/min; column temperature: 40 °C). The detection system consisted of a model 5100A Coulochem detector equipped with a 5021 conditioning cell and a 5011 high sensitivity cell (ESA, Bedford, MA). Potentials for the conditioning cell and detectors 1 and 2 were -0.20, +0.08 and +0.45 V, respectively (gain: 30x100). Quantification was done by measuring peak heights. The lower detection limit for 5-HT was estimated at 5 nM. In samples in which the eicosanoids or 5-HT could not be detected, the concentration was assumed to equal the lower detection limit concentration.

Histological examination

Thirteen coronary artery segments obtained from 6 hearts, which were used for a control concentration response curve by untreated platelets, were analyzed histologically to correlate the functional results to signs of early atherosclerosis. After the organ bath experiment the vessel segments were fixed in 10% formalin, and stained with haematoxylin-eosin and elastic-Van Gieson. Sections were examined microscopically, and signs of atherosclerosis were scored in two blind independent sessions in a semi-quantitative fashion, modified from Ginsburg et al. (1984) [17]. In brief, three vessel characteristics were scored: degree of luminal occlusion, fragmentation of the internal elastic lamina and intimal hyperproliferation. Each category was scored on a scale from 1 to 3, with 1 being completely healthy and 3 being severely diseased. Thus, the mean Coronary Artery Disease Scale (CADS) was calculated for each vessel segment and correlated to functional parameters.

Analysis of data

All data are presented as mean ± SEM. Analysis of variance for multiple comparisons (Duncan) followed by a Student's *t*-test, for paired data where appropriate, were used

for comparison of mean contractile responses and organ bath concentrations. A correlation coefficient, *r*, was calculated according to Pearson. A *p* value less than 0.05 was assumed to denote a significant difference.

Compounds used in the study

The following compounds were used during the organ bath experiments: ketanserin tartrate (gift: Janssen Pharmaceutica, Beerse, Belgium); prostaglandin $F_{2\alpha}$ (tris salt) and substance P acetate (Sigma Chem. Co., St. Louis, USA); SQ30741 (1S-[1<a,2<a(Z),3<a, 4<a]-7-[3-[[[[(1-oxoheptyl)amino]acetyl]amino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]- 5-heptenoic acid; gift: Bristol-Myers Squibb, Princeton, New Jersey, USA). All compounds were dissolved in distilled water except SQ30741, which was dissolved in ethanol.

6.3 Results

Responses of coronary artery segments

Mean relaxation to substance P was 74 ± 8% of precontraction to $PGF_{2\alpha}$ (1 μM), which is in accordance with previous studies on human coronary arteries obtained from heart transplant recipients [18]. Potassium (100 mM) caused a mean contractile response of 55.5 ± 7.6 mN. No difference in the mean contractile response to potassium (100 mM) was observed in any of the groups compared.

Platelets contracted the vessel segments in a concentration-dependent manner. No contractile or relaxant response was elicited by the citrate buffer itself in which the platelets were suspended (data not shown). At the highest platelet concentration tested, the concentration response curve had not reached the maximum response (Figure 1). Since one is restricted by the amount of blood that can be drawn from platelet-donors, higher platelet concentrations were not used. The maximal response at 3.10^{10} platelets/l reached 28.4 ± 7.1% of the response to potassium (100 mM). Both ketanserin (1 μM, a $5\text{-}HT_2$ receptor antagonist) and SQ30741 (0.01 μM, a TxA_2 receptor antagonist) reduced ($p<0.05$) the maximal response to platelets to 8.8 ± 2.3% and 3.2 ± 2.2% of potassium-induced responses, respectively. A combination of these two compounds practically abolished the contractile response and even caused platelets to evoke small relaxations in some vessel segments (relaxation: 0.9 ± 1.9% of potassium-induced responses, Figure 1).

During the period in which the platelet-donors took aspirin (range 7-13 days) no

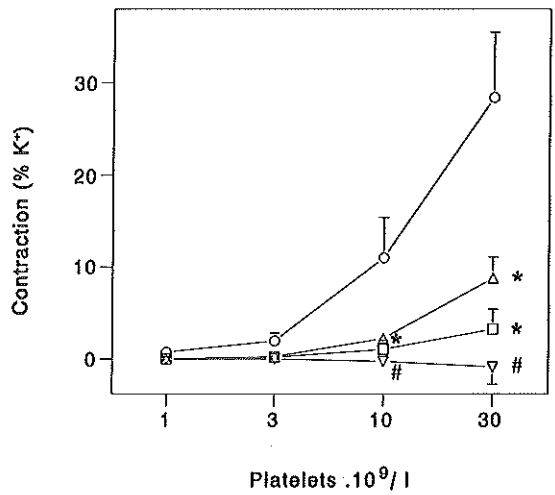

Figure 1. Contraction of the human isolated coronary artery to activated platelets. Control concentration response curve:○ , curve in the presence of ketanserin (1 μM):△ ; curve in the presence of SQ30741 (0.01 μM):□ ; curve in the presence of the combination of ketanserin (1 μM) and SQ30741 (0.01 μM):▽ ; n=6 each. *: different ($p<0.05$) from control response, #: different ($p<0.05$) from the control response and also from the response to platelets in the presence of ketanserin alone. At a concentration of 10^{10} platelets/l, the response to platelets in the presence of SQ30741 alone was found to be different ($p<0.05$, *) from the control curve, whereas the contractile response to platelets in the presence of the combination of ketanserin and SQ30741 was different ($p<0.05$, #) from both the control response and from the response in the presence of ketanserin alone. Where no error bar is visible, error falls within the limits of the symbol.

adverse effects were observed. The contractile response to platelets obtained after aspirin treatment was significantly decreased reaching only a maximum of 7.6 ± 2.7% of potassium-induced contraction (Figure 2). Although SQ30741 (0.01 μM) was more potent than ketanserin (1 μM) against untreated platelets (Figure 1), ketanserin appeared to be more potent than SQ30741 against platelets from aspirin-treated platelet donors. As depicted in Figure 3, SQ30741 (0.01 μM) no longer significantly reduced the residual contractile responses. Contractions to aspirin-treated-platelets were however significantly attenuated by ketanserin (1 μM) (contraction: 0.9 ± 1.9% of potassium-induced

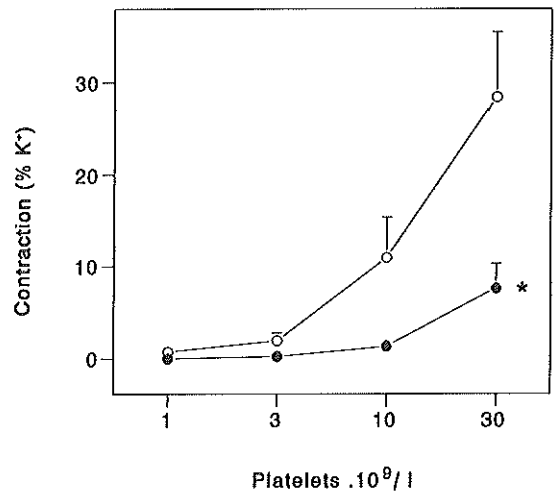

Figure 2. Contraction of the human isolated coronary artery to activated platelets. Concentration response curve of platelets obtained from subjects before aspirin treatment:○; concentration response curve of platelets obtained from the same subjects after treatment with 40 mg/day aspirin:● ; n=6 each. Contractions are expressed as a percentage of contraction induced by 100 mM potassium. *, different ($p<0.05$) from 'non-treated' (control) response. Where no error bar is visible, it falls within the limit of the symbol.

contraction). Pretreatment with a combination of ketanserin and SQ30741 now caused a clear concentration dependent platelet-induced relaxation of the coronary artery segments (-5.0 ± 1.8% of the potassium-induced contractile effect, Figure 3).

Concentrations of eicosanoids and 5-HT in the organ bath solution

The concentrations of eicosanoids (TxB_2, PGE_2, 6-keto-$PGF_{1\alpha}$) and 5-HT in the organ bath solution are shown in Table 1. When citrate buffer without platelets was added to the organ bath, no PGE_2, 6-keto-$PGF_{1\alpha}$ or 5-HT could be measured significantly different from the lower detection limits (Table 1; Section A). The concentration of TxB_2 in these organ baths however, was significantly higher than the lower detection limit. After addition of 3.10^{10} non-aspirin-treated-platelets/l (Table 1; Section B) a significant increase of TxB_2, PGE_2, and 5-HT was measured. The concentration of 6-keto-$PGF_{1\alpha}$ increased

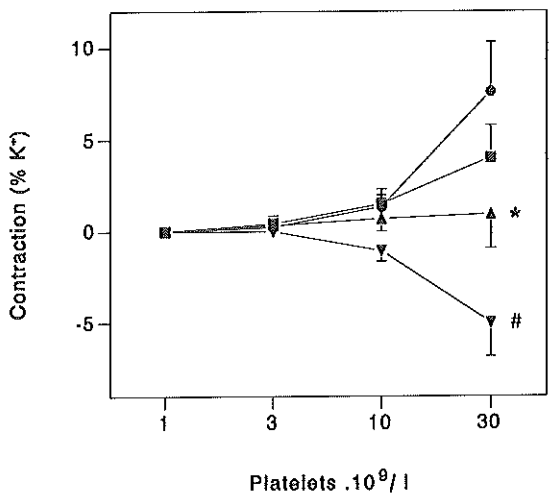

Figure 3. Contraction of the human isolated coronary artery to activated, aspirin treated platelets. Control concentration response curve:●; curve in the presence of ketanserin (1 μM):▲ ; curve in the presence of SQ30741 (0.01 μM):■ ; curve in the presence of the combination of ketanserin (1 μM) and SQ30741 (0.01 μM):▼ ; n=6 each. Contractions are expressed as a percentage of contraction induced by 100 mM potassium. *: different ($p<0.05$) from control response. #, different ($p<0.05$) from the control response and also from the response to platelets in the presence of SQ30741 alone. Where no error bar is visible, it falls within the limit of the symbol.

from below the lower detection limit to 0.29 ± 0.08 nM. Ketanserin or SQ30741 did not influence the amount of TxB_2, PGE_2, 6-keto-$PGF_{1\alpha}$ or 5-HT in the organ baths. Platelets obtained from aspirin-treated platelet-donors (Table 1; Section C) secreted significantly smaller amounts of TxA_2 in the organ baths, as measured by the concentration of TxB_2. The amount of PGE_2, 6-keto-$PGF_{1\alpha}$ or 5-HT in the organ bath was not significantly affected by the treatment with aspirin.

Coronary Artery Disease Scale (CADS) and functional parameters

Histological examination of the control vessel segments used for experiments with untreated platelets, revealed early signs of atherosclerosis: modest intimal hyperproliferation, internal elastic lamina fragmentation, and luminal occlusion. The mean

CADS varied from 1.15 in a vessel segment obtained from a 7 year old tissue donor, to 2.00 in a segment obtained from a 47 year old tissue donor. The CADS correlated positively with age (r=0.84, p=0.000), but negatively with the percentage of relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ (1 μM) (r=-0.67, p=0.006). The CADS also correlated negatively with the contractile response to untreated platelets (3.10^{10}/l), both when expressed as a percentage of potassium (100 mM)-induced contractions (r=-0.60, p=0.01) and also when expressed as contraction in mN (r=-0.63, p=0.01).

6.4 Discussion

In the present study we showed that both a TxA_2 receptor antagonist and a 5-HT_2 receptor antagonist or low dose aspirin, taken by the platelet-donors, can attenuate platelet-induced contractions of human isolated coronary arteries. A 5-HT_2 receptor antagonist caused a significant further reduction of the residual contractile response caused by aspirin treated-platelets. A TxA_2 receptor antagonist caused only minor, non-significant further attenuation of contractions induced by the aspirin treated-platelets.

The effect of a TxA_2 receptor antagonist

Platelets have been described to contract human isolated blood vessels by releasing 5-HT and TxA_2, whereas platelet-derived ADP caused both the human coronary artery and the internal mammary artery to relax via the induction of endothelium-derived relaxing factor (nitric oxide, NO) [3, 4]. The thromboxane synthase inhibitor dezmegrel, however, only slightly enhanced platelet-induced relaxations of the precontracted ($PGF_{2\alpha}$) human coronary artery. The blockade of thromboxane synthase by dezmegrel resulted in an increased synthesis by platelets of both $PGF_{2\alpha}$ and PGE_2, which were found to take over a part of the contractile effect of TxA_2. This seemed to be a major limitation to the clinical efficacy of thromboxane synthase inhibitors [3]. More potent antagonism of platelet-induced contractions was achieved with the TxA_2 receptor antagonist SQ30741 [19], which, in a concentration of 0.01 μM, caused marked inhibition of platelet-induced contraction of the human internal mammary artery and the human saphenous vein [4]. The present experiments show this to be valid also for the effect of untreated platelets on the human isolated coronary artery (Figure 1). Although another TxA_2 receptor antagonist (GR32191B) has been shown to be ineffective in the prevention of coronary restenosis

Table 1. Concentration of TxB_2, PGE_2 and 6-keto-$PGF_{1\alpha}$ and 5-HT (all in nM), measured in samples taken from the organ bath, 30 min after adding citrate buffer with (B and C) or without (A) platelets.

	TxB_2	PGE_2	6-keto-$PGF_{1\alpha}$	5-HT
A. Citrate buffer, no platelets	0.39 (0.01)†	0.07 (0.00)*	0.14 (0.00)	5.90 (0.48)*
B. Platelets from non-treated platelet-donors				
Control	2.19 (0.43)	0.42 (0.16)	0.29 (0.08)	65.17 (9.94)
Ketanserin, 1 μM	2.30 (0.43)	0.44 (0.13)	0.23 (0.05)	76.32 (12.38)
SQ30741, 0.01 μM	2.15 (0.59)	0.46 (0.14)	0.19 (0.03)	56.25 (7.09)
Ketanserin, 1 μM, and SQ30741, 0.01 μM	1.70 (0.26)	0.42 (0.16)	0.23 (0.06)	79.00 (12.77)
C. Platelets from aspirin-treated platelet-donors				
Control	0.66 (0.05)‡	0.33 (0.17)	0.54 (0.26)	64.03 (8.98)
Ketanserin, 1 μM	0.56 (0.07)	0.40 (0.30)	0.40 (0.24)	N.D.
SQ30741, 0.01 μM	1.04 (0.55)	0.42 (0.27)	0.81 (0.37)	N.D.
Ketanserin, 1 μM, and SQ30741, 0.01 μM	0.42 (0.05)	0.18 (0.08)	0.34 (0.19)	N.D.

N.D.: Not determined; For statistical evaluation, the measured concentrations in the presence or absence of an antagonist or platelets (buffer, A) were compared to the respective (aspirin-untreated (B) or -treated (C)) control situation where no antagonist was present. *: significantly different from the concentration measured for control platelets; †: as * but also significantly different from the detection limit for this compound; The aspirin-treated control situation was compared to the non-treated situation: ‡:significantly different from the untreated control situation. In samples in which the eicosanoid or 5-HT could not be detected, the concentration was assumed to equal the lower detection limit concentration.

after percutaneous transluminal coronary angioplasty (PTCA) [20], inhibition of the TxA_2 receptor may still have clinical perspective in the prevention of coronary vasospasm since they undoubtedly have potent receptor antagonist activity and do not affect the production of PGI_2 and PGE_2 [21]. On the other hand, high concentrations of TxA_2, which may be present close to the vascular smooth muscle cell, could displace a competitive TxA_2 receptor antagonist, limiting the clinical efficacy of this potentially new class of anti-platelet drugs [22].

The effect of 5-HT receptor antagonism

Ketanserin (1 μM), a 5-HT_2 receptor antagonist, caused significant attenuation of platelet-induced contractions, indicating that the 5-HT_2 receptor plays an important role in the contractile response, as was found previously for both the human isolated internal mammary artery and the saphenous vein [4]. The contractile response to exogenous 5-HT is mediated by a mixed population of 5-HT_1-like and 5-HT_2 receptors, but predominant mediation by 5-HT_2 receptors has been reported [23, 24]. It is quite likely, however, that the 5-HT_1-like receptor also plays a significant role. First, ketanserin alone was not found to be effective in the prevention of vasospastic angina [25]. Secondly, one may expect that 5-HT in low concentrations (less than 1 μM) activates especially 5-HT_1-like receptors since it has significantly higher affinity for the different 5-HT_1 receptor subtypes than for the 5-HT_2 receptor [26]. Indeed, several authors have suggested that a mixed 5-HT_1-like - 5-HT_2 receptor antagonist may be more effective in the treatment of coronary vasospasm [27-30].

The effect of low dose aspirin

The mechanism by which aspirin decreases the morbidity and mortality to cardiovascular disease is believed to be a generalized inhibition of production of contractile cyclooxygenase products, such as PGH_2, TxA_2 and PGE_2. This process can be counteracted by a concurrent aspirin-induced decrease of the production of vasorelaxant and antiaggregatory PGI_2 [21, 31]. The present experiments show that the systemic use of low dose aspirin results in a relatively selective decrease of TxA_2 production. The production of PGE_2 was left almost unaltered (Table 1). Although the vessel segments in our study had not been exposed to aspirin, this same dose has previously been shown to preserve prostacyclin production in humans *in vivo* [5, 6]. 6-Keto-$PGF_{1\alpha}$ could only be detected after addition of platelets to the organ bath, indicating a, presumably endothelial, response to platelets or platelet products and the absence of a basal prostacyclin production *in vitro* [32].

TxB_2 was detected in the organ bath even when no platelets had been added, indicating basal vascular production of TxA_2, although TxA_2 derived from leftover tissue-donor platelets cannot entirely be excluded. The concentration of 5-HT was left unchanged by the treatment with low dose aspirin. Apart from HPLC measurements this could also be reasoned from the fact that ketanserin was still significantly active as an antagonist after treatment of the platelets with aspirin (Figure 3), whereas SQ30741 caused only minor, non-significant additional attenuation of the residual contractile response. This was presumably due to a decrease of the TxA_2-induced part of the platelet-induced contractile response caused by treatment with aspirin.

Correlation of Coronary Artery Disease Score (CADS) and functional parameters

Despite the fact that the coronary arteries were obtained from relatively young, apparently healthy organ donors, most vessel segments showed modest, age related signs of atherosclerosis: intimal hyperproliferation, internal elastic lamina fragmentation and luminal occlusion. It has to be kept in mind, however, that we avoided the use of vascular segments with distinct, macroscopically visible, atherosclerotic lesions. This analysis therefore refers to early atherosclerotic development only. On the other hand, the present CADS appears to be in line with Ginsburg et al. (1984) [17], who found that CADS in coronary arteries ranged from 1.5 between 10 and 20 years of age, up to 2.2 between 40 and 50 years of age. We observed that relaxation to substance P was inversely correlated with the development of early atherosclerosis, confirming earlier reports concluding that atherosclerosis reduces the endothelium dependent, nitric oxide-mediated, relaxant response to substance P in the human isolated coronary artery [18, 33].

In contrast to some animal models [34, 35], we observed a tendency towards a decreased platelet-induced contractile response in mildly diseased vessel segments. In the present set-up, both the luminal and serosal side were exposed to the platelet products. Kaul and co-workers (1992) studied a perfusion model in which platelets were added on either the luminal or the serosal side. Indeed, only intraluminal, and not abluminal activation of platelets resulted in different responses of atherosclerotic and normal perfused arteries [34]. It would therefore be of great interest to develop a similar model for the human isolated coronary artery. Furthermore it has to be noted that different platelet-stimulating agonists may induce the release of a somewhat different spectrum of platelet products [11]. In this model, as well as in previously adopted models [3, 4, 10], platelets are apparently activated upon exposure to tissue collagen and Ca^{2+} in the Krebs solution. Thus, despite the human

nature of both platelets and coronary arteries, and *in vivo* application of low dose aspirin, care has to be taken when extrapolating results obtained *in vitro*, to the *in vivo* situation.

Interactions between platelet products

5-HT and TxA_2 are known to take part in an amplifying interaction at the vascular smooth muscle level [36-38]. Therefore, it seems rational not only to counteract the contractile effects of both TxA_2 and 5-HT per se, but also to interfere with the amplifying interaction. Interestingly, the presence of the stable TxA_2 mimetic, U46619, increased the response mediated by 5-HT_1-like receptors but not by 5-HT_2 receptors [39, 40], emphasizing also in this respect the potentially beneficial effect of a mixed 5-HT_1-like / 5-HT_2 receptor antagonist when compared to a selective 5-HT_2 receptor antagonist like ketanserin.

Whether the effect of TxA_2 *in vivo* could be counteracted more effectively by a thromboxane synthase inhibitor, TxA_2 receptor antagonists or a combined synthase inhibitor-receptor antagonist than by low dose aspirin still remains to be proven [22], since low dose aspirin is clinically very effective at low cost [1, 8, 9]. In conclusion, the present study shows that additional antagonism of the coronary artery contractile 5-HT receptors may increase the efficacy of low dose aspirin *in vivo*.

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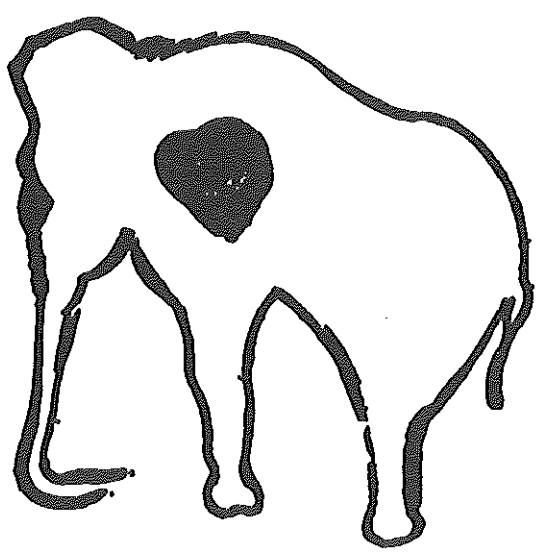

Primitive sketch of an elephant's heart. Was painted 25.000 years ago in the El Pindal Caves, Spain

Part 3

Peptides

Chapter 7

Endothelin receptors in the human coronary artery, ventricle and atrium; a quantitative autoradiographic analysis*

Summary - In the present experiments we investigated endothelin (ET) receptors in the human coronary artery, and in ventricular and atrial muscle using quantitative receptor autoradiography. Displacement of [^{125}I]Sf6b (Sarafotoxin S6b) (30 pM)- and [^{125}I]ET-1 (30 pM)-labelled binding sites was studied using ET-1, the ET_A receptor selective ligand BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]), and the ET_B receptor selective ligand [Ala^{1,3,11,15}]ET-1.

Specific binding was more dense in atrium and coronary artery (relative optical density (r.o.d.): 0.14 ± 0.01 and 0.16 ± 0.01, respectively) than in ventricular muscle (r.o.d.: 0.10 ± 0.01). In the coronary artery, binding was especially dense in the media. ET-1 displaced [^{125}I]ET-1 and [^{125}I]Sf6b monophasically in atrium, ventricle and coronary artery. [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1 and [^{125}I]Sf6b-labelled sites biphasically in the ventricle and in the atrium. In the human coronary artery, [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1-labelled sites monophasically (pIC_{50}: ET-1 (9.72 ± 0.12) > BQ-123 (6.84 ± 0.08) > [Ala^{1,3,11,15}]ET-1 (6.40 ± 0.12)). By contrast, [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]Sf6b-labelled coronary artery sites biphasically (high affinity pIC_{50}: BQ-123, 9.03 ± 0.25; [Ala^{1,3,11,15}]ET-1, 8.40 ± 0.14; low affinity pIC_{50}: BQ-123, 7.24 ± 0.14; [Ala^{1,3,11,15}]ET-1, 6.99 ± 0.09). These data indicate that both [^{125}I]ET-1 and [^{125}I]Sf6b-labelled ET_A and ET_B binding sites in human ventricular and atrial muscle. In the human coronary artery, both radioligands labelled ET_A binding sites, but [^{125}I]Sf6b also labelled a non-ET_A, non-ET_B binding site with relatively high affinity for both BQ-123 and [Ala^{1,3,11,15}]ET-1.

*, *Based on:* Bax, W.A., Bruinvels, A.T., Van Suylen, R.J., Saxena, P.R. and Hoyer, D. (1993) Endothelin receptors in the human coronary artery, ventricle and atrium; A quantitative autoradiographic analysis. *Naunyn Schmiedeberg's Arch. Pharmacol.* **348**, 403-410.

7.1 Introduction

Endothelin (ET) was originally described as a potent vasoconstrictor peptide [1]. In addition, Wright and Fozard described a vasodilatory effect [2], later found to be mediated, at least partially, by an endothelium-dependent mechanism involving the release of nitric oxide (NO [3]) and/or prostacyclin (PGI_2 [4]). In the heart, endothelin has been reported to elicit both positive inotropic [5-7] and chronotropic [8] effects. Furthermore, endothelin induced mitogenesis of vascular smooth muscle cells [9, 10] and hypertrophy of myocardial cells [11] indicating that endothelin may play a role in the development of atherosclerosis and cardiac hypertrophy.

Two distinct endothelin receptor subtypes (ET_A [12] and ET_B [13]) have been cloned. In radioligand binding studies, the ET_A receptor has somewhat higher affinity for ET-1 in comparison with isopeptides like ET-3 or sarafotoxin S6b. The ET_B receptor has almost equal affinity for all endothelins and sarafotoxin S6b [12-14]. The development of selective ligands that distinguish between these receptors has helped to characterize the receptors present in various tissues such as the lungs [15], kidneys [16] and the myocardium [17]. In the diseased human heart obtained from heart transplant recipients, both ET_A and ET_B receptors have been described in the atrium and ventricle, and in the atrioventricular conducting system, using in situ hybridization and radioligand binding applying the ET_B receptor selective [^{125}I]BQ3020, [^{125}I][Ala^{1,3,11,15}]ET-1, and the relatively nonselective [^{125}I]ET-1 as radioligands [17-19].

In the present study we characterized endothelin receptors in the human coronary artery obtained from healthy and diseased hearts. For comparison we also studied endothelin receptors in the human ventricle and human atrium. We used the ET_A receptor selective ligand BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]) [20], and the ET_B receptor selective ligand [Ala^{1,3,11,15}]ET-1 [15], and ET-1, to displace binding of the relatively non-selective ligands [^{125}I]ET-1 and [^{125}I]Sf6b. A part of this study was presented to the British Pharmacological Society [21].

7.2 Materials and methods

Preparation of the tissue

A total of 16 human hearts was used in the present investigation. All experiments were approved by the Ethical Committee of the University Hospital Rotterdam 'Dijkzigt', The Netherlands. The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services / Eurotransplant Foundation, Leiden, The Netherlands) after removal of the aortic and pulmonary valves for valve transplantation. Twelve of the hearts were obtained from patients who had died of non-cardiac disorders (6 cerebrovascular accident, 6 polytrauma; 7 male, 5 female; age 7-55 yrs). These hearts were stored at 0-4 °C in a sterile organ protecting solution immediately following circulatory arrest and arrived in the laboratory within 20 h after cardiac arrest. Four hearts were obtained from patients undergoing cardiac transplantation (2 dilating cardiomyopathy, included in the groups of cardiac atrium and ventricle and 2 end-stage coronary artery disease, included in the coronary artery group; all male; age 41-55 yrs). After arrival in the laboratory, the right and left descending coronary artery and samples of free wall of right atrium and left ventricle (± 1.5 cm^3) were removed and the vessels were cleaned of fat and connective tissue. All samples were embedded in M-1 embedding matrix (Lipshaw, Pittsburgh PA, USA) and frozen in liquid nitrogen. The tissue was kept at -70 °C until further use. Serial sections of 10 µm were cut on a micro-cryostat and thaw-mounted on gelatin-coated glass slides and kept at -20 °C until binding experiments.

Autoradiographic protocol

The slides were thawed and subsequently preincubated for 15 min in a Tris-BSA buffer, pH: 7.4. (Tris: tris(hydroxymethyl)-aminomethan); BSA: Bovine Serum Albumin (both from Sigma Chemical Co., St. Louis MO, USA). The tissue was then incubated, at room temperature, for 120 min in 10 ml Tris-BSA buffer containing either 30 pM [^{125}I]ET-1 or 30 pM [^{125}I]Sf6b. The incubation buffer also contained chymostatin (2 mg/l; Bachem, Bubendorf, Switzerland), leupeptin (4 mg/l; Sigma Chemical Co., St. Louis MO, USA), bacitracin (40 mg/l; Sigma, Buchs, Switzerland) and 5 mM $MnCl_2$. Non-specific binding was assessed in the presence of 0.3 µM unlabelled ET-1. Displacement of radioligand binding was studied by adding unlabelled ET-1 (concentration range 1 pM - 0.3 µM), [Ala^{1,3,11,15}]ET-1 (concentration range 30 pM - 10 µM) or BQ-123 (concentration range 3 pM - 30 µM) to the radioligand containing buffer. After incubation, the slides were rinsed twice in ice-cold buffer for 5 min. The slides were then rapidly dried by

exposing to a stream of cold air and subsequently apposed to [^{3}H]Hyperfilm (Amersham, Buckinghamshire, UK) for 6 h. After exposure the films were developed (Kodak D19, Rochester, New York, USA), fixated and dried. In addition, we performed high resolution autoradiography by placing emulsion-coated (Kodak NTB_3, Rochester, New York, USA) glass coverslips to the slides and exposed for 18 h. The coverslip slides were developed (Kodak D19, Rochester, New York, USA) and fixated. Subsequently the tissue was stained with elastic-Van Gieson. Experiments (n=7-14) were performed in two separate sessions.

Analysis of the data

Binding on film was quantified densitometrically using a computerized image analysis system (MCID Imaging Research, St Catherines, Ontario, Canada). Displacement curves were analyzed for individual segments, generating competition curves for a one or two site-model, depending on the best fit (based on the χ^2 values, Grafit, Erithacus Software Ltd., Staines, UK). The slope factor of the displacement curves by [$Ala^{1,3,11,15}$]ET-1 and BQ-123 in the coronary artery were calculated according to Ref. 22, by fitting the curve in a one-receptor model.

Compounds

[^{125}I]ET-1 and [^{125}I]Sf6b (both with specific activity of 2200 Ci/mmol) were purchased from ANAWA (Zürich, Switzerland). ET-1, [$Ala^{1,3,11,15}$]ET-1 and BQ-123 were purchased from Neosystem S.A. (Strasbourg, France).

7.3 Results

Specific binding of [^{125}I]Sf6b and [^{125}I]ET-1 in ventricular and atrial muscle was homogeneously distributed over the tissue and specific binding was more dense in the atrium and the coronary artery (relative optical density (r.o.d.): 0.14 ± 0.01 and 0.16 ± 0.01 respectively) than in the ventricle (r.o.d.: 0.10 ± 0.01; Figure 1). In the coronary artery, binding on film consisted exclusively of binding to the media, but coverslip high resolution autoradiography revealed also a signal in a thin adventitial layer and between media and the intimal hyperplasia. Labelling in the latter two regions of the vessel consisted mainly of non-specific binding, and did not show on [^{3}H]Hyperfilm (Figure 2).

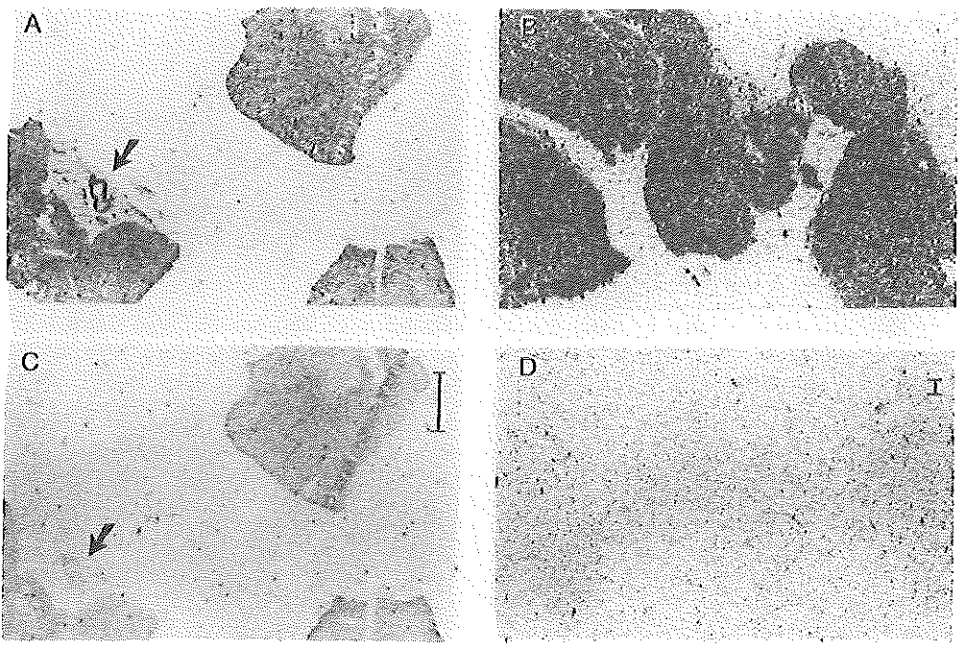

Figure 1. Autoradiographs of total (A and B) and non-specific binding (C and D) of [^{125}I]ET-1-labelled ventricular segments (three different patients, A and C) and one [^{125}I]ET-1-labelled atrial segment (B and D). Note differences in receptor density between ventricle on one hand and atrium and coronary artery (embedded in fat attached to the left ventricle: A and C, arrow) on the other hand. Scale bars: panel C, 4 mm; panel D, 0.5 mm.

ET-1 displaced [^{125}I]Sf6b and [^{125}I]ET-1 monophasically in the ventricle (Figure 3; pIC_{50} values of the curves are shown in Table 1). [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1 and [^{125}I]Sf6b-labelled sites biphasically in the ventricle. Assuming a two receptor model to be present, the proportion of high-affinity sites of BQ-123 (which should equal the proportion of low affinity sites of [Ala^{1,3,11,15}]ET-1) as compared to the high affinity sites of [Ala^{1,3,11,15}]ET-1 (which should equal the proportion of low affinity sites of BQ-123) was 53 ± 3% : 47 ± 3% in the ventricle (percentages for separate curves: see Table 1).

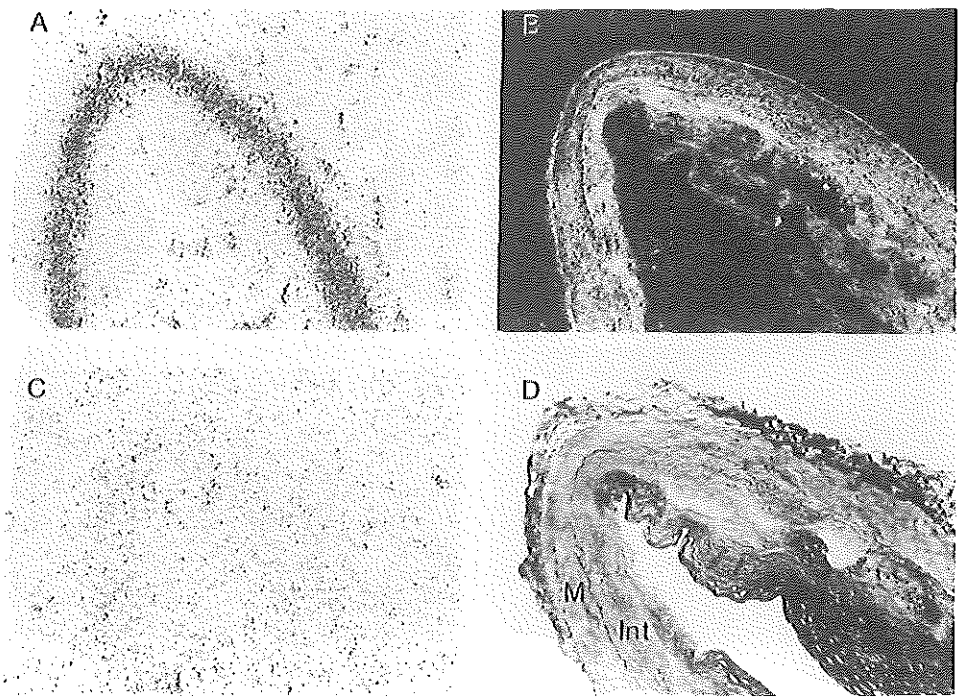

Figure 2. Left panels: Autoradiography film of total (A) and non-specific (C) binding of one [^{125}I]ET-1-labelled coronary artery segment, obtained from a patient undergoing a cardiac transplantation for ischaemic coronary artery disease. Right panels: High resolution autoradiography (C) and light microscopy (elastic-Van Gieson staining, 40x, D) of the same coronary artery segment. Int, intima: a combination of intimal hyperplasia and intimal fibrosis; M, media.

Also in the atrium [^{125}I]Sf6b and [^{125}I]ET-1 were displaced monophasically by ET-1, but biphasically by [Ala^{1,3,11,15}]ET-1 and BQ-123 (Figure 4; pIC_{50} values of the curves are shown in Table 2). In the atrium, the proportion of high affinity sites of BQ-123 as compared to the high affinity sites of [Ala^{1,3,11,15}]ET-1 was 68 ± 3% : 32 ± 3% (percentages for separate curves: see Table 2).

In the human coronary artery, ET-1 displaced both [^{125}I]Sf6b and [^{125}I]ET-1 in a monophasic manner. [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1-labelled sites monophasically in 6 and 7 segments respectively, out of 7 individually analyzed tissue segments. In contrast, [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]Sf6b-labelled sites biphasically in 9 segments each, out of 14 individually analyzed coronary artery segments.

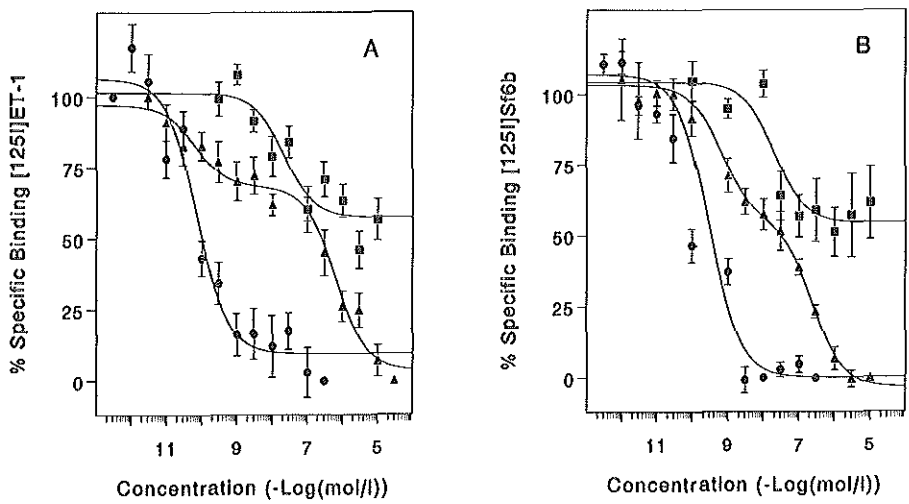

Figure 3. Human left ventricle. Displacement (% of total specific binding) of [^{125}I]ET-1 (panel A) and [^{125}I]Sf6b (panel B) by ET-1 (●), BQ-123 (■) and [Ala^{1,3,11,15}]ET-1 (▲).

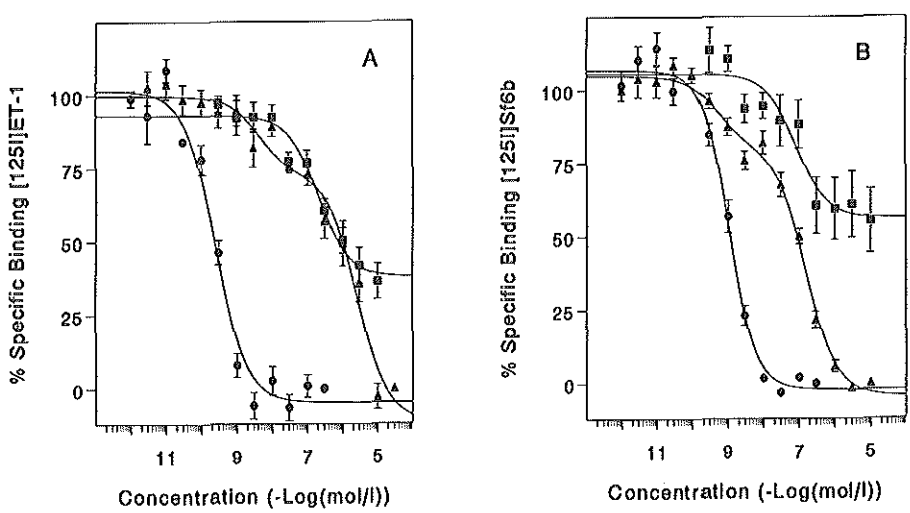

Figure 4. Human right atrium. Displacement (% of total specific binding) of [^{125}I]ET-1 (panel A) and [^{125}I]Sf6b (panel B) by ET-1 (●), BQ-123 (■) and [Ala^{1,3,11,15}]ET-1 (▲).

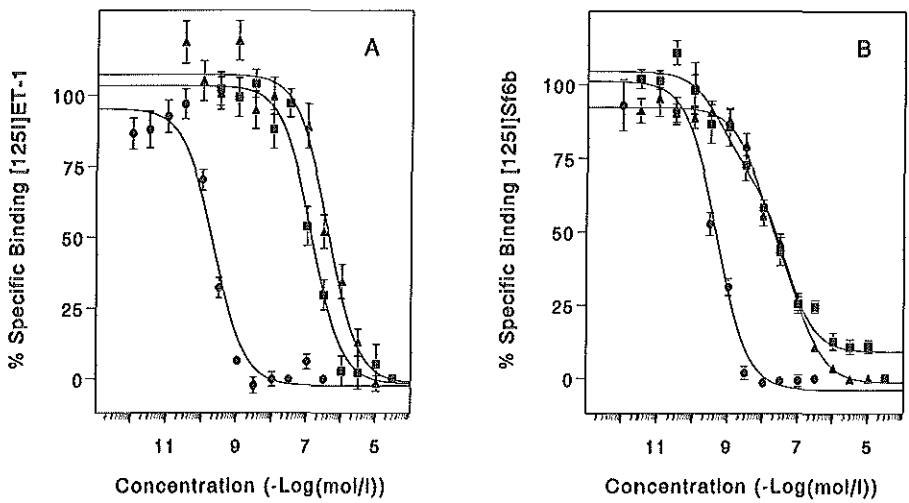

Figure 5. Human coronary artery. Displacement (% of total specific binding) of [^{125}I]ET-1 (panel A) and [^{125}I]Sf6b (panel B) by ET-1 (●), BQ-123 (■) and [Ala^{1,3,11,15}]ET-1 (▲).

Also analysis of the mean curves in the coronary artery revealed a monophasic curve as the best fit for the displacement of [^{125}I]ET-1 by [Ala^{1,3,11,15}]ET-1 and BQ-123, whereas the displacement of [^{125}I]Sf6b by [Ala^{1,3,11,15}]ET-1 and BQ-123 were both best described by a biphasic curve (Figure 5). In addition, the slope factor (or pseudo Hill number) of the displacement curve of [^{125}I]Sf6b by [Ala^{1,3,11,15}]ET-1 or BQ-123, when fitted to a monophasic model according to De Lean et al. (1978), was significantly lower (0.64 ± 0.09 and 0.69 ± 0.11 respectively) than the slope of the displacement curve by [Ala^{1,3,11,15}]ET-1 or BQ-123 of [^{125}I]ET-1 (0.95 ± 0.08) and 1.27 ± 0.14 respectively). The [^{125}I]Sf6b-labelled proportion of receptors with high affinity for both BQ-123 and [Ala^{1,3,11,15}]ET-1 was 49 ± 4% : 51 ± 4% when compared to the proportion of [^{125}I]Sf6b-labelled- sites with 'low' affinity for BQ-123 and [Ala^{1,3,11,15}]ET-1. We observed no differences in receptor density, affinity values or slope factors between healthy and diseased hearts. Therefore, the data refer to the mean of all ventricle, atrium or coronary artery samples.

Table 1. Human ventricle. pIC_{50} (-Log IC_{50}) for the monophasic displacement by ET-1, and in case of biphasic displacement the high (pIC_{50} high) and/or low (pIC_{50} low) affinity value and proportion (% high, % low) of the affinity phases of [Ala^{1,3,11,15}]ET-1 and BQ-123.

		[^{125}I]ET-1	[^{125}I]Sf6b
ET-1	pIC_{50}[1]	10.16 ± 0.08	9.49 ± 0.08
[Ala^{1,3,11,15}]ET-1	pIC_{50} high	9.59 ± 0.34	9.48 ± 0.16
	% high	39.7 ± 5.1	50.6 ± 5.1
	pIC_{50} low	6.05 ± 0.20	6.54 ± 0.12
	% low	60.3 ± 5.1	49.4 ± 5.1
BQ-123	pIC_{50} high[2]	7.04 ± 0.24	7.49 ± 0.10
	% high[2]	50.1 ± 5.6	49.4 ± 14.3

[1], Monophasic complete displacement;
[2], Lower affinity phase could not be analyzed because plateau was reached.

Table 2. Human atrium. pIC_{50} (-Log IC_{50}) for the monophasic displacement by ET-1, and in case of biphasic displacement the high (pIC_{50} high) and/or low (pIC_{50} low) affinity value and proportion (% high, % low) of the affinity phases of [Ala^{1,3,11,15}]ET-1 and BQ-123.

		[^{125}I]ET-1	[^{125}I]Sf6b
ET-1	pIC_{50}[1]	9.61 ± 0.04	8.95 ± 0.07
[Ala^{1,3,11,15}]ET-1	pIC_{50} high	9.05 ± 0.33	9.36 ± 0.09
	% high	27.9 ± 5.1	24.7 ± 2.7
	pIC_{50} low	5.79 ± 0.17	6.91 ± 0.05
	% low	72.1 ± 5.1	75.3 ± 2.7
BQ-123	pIC_{50} high[2]	6.67 ± 0.12	7.45 ± 0.40
	% high[2]	58.7 ± 5.5	48.3 ± 13.2

[1], Monophasic complete displacement;
[2], Lower affinity phase could not be analyzed because plateau was reached.

Table 3. Human coronary artery. pIC_{50} (-Log IC_{50} (mol/l)) for the monophasic displacement (pIC_{50} mono) or, in case of biphasic displacement, the high (pIC_{50} high) and low (pIC_{50} low) affinity value and proportion (% high, % low) of the affinity phases of [$Ala^{1,3,11,15}$]ET-1 and BQ-123. Slope factor of the curves (or pseudo Hill number), see Ref. 22.

		[^{125}I]ET-1	Slope factor	[^{125}I]Sf6b	Slope factor
ET-1	pIC_{50}	9.72 ± 0.02[1]		9.35 ± 0.04	
[$Ala^{1,3,11,15}$]ET-1	pIC_{50} mono	6.40 ± 0.12[1]	0.95 ± 0.08	7.66 ± 0.06[2]	0.64 ± 0.09
	pIC_{50} high			8.40 ± 0.14	
	% high			47.3 ± 4.8	
	pIC_{50} low			6.99 ± 0.09	
	% low			52.7 ± 4.8	
BQ-123	pIC_{50} mono	6.84 ± 0.08[1]	1.27 ± 0.14	7.82 ± 0.13[2]	0.69 ± 0.11
	pIC_{50} high			9.03 ± 0.25	
	% high			50.1 ± 6.1	
	pIC_{50} low			7.24 ± 0.14	
	% low			49.9 ± 6.1	

[1], Monophasic complete displacement;
[2], pIC_{50} value when fitted to a monophasic model to calculate the slope factor of the curve.

7.4 Discussion

Both [^{125}I]ET-1 and [^{125}I]Sf6b were found to label a mixed population of binding sites in human ventricle and atrium. Using [^{125}I]ET-1, we found an apparently single receptor population in the human coronary artery with characteristics similar to one of the two receptors in the ventricle and atrium. However, the radioligand [^{125}I]Sf6b also seemed to label a coronary artery site with binding characteristics different from the atrial and ventricular endothelin receptors. It is to be noted that non-specific binding was defined using unlabelled ET-1. In general, non-specific binding should preferably be determined using a cold ligand, different from the radioligands [^{125}I]ET-1 and [^{125}I]Sf6b. However, little is known about truly non-selective endothelin receptor ligands. Furthermore, the maximum displacement by [$Ala^{1,3,11,15}$]ET-1 and ET-1 was similar for both [^{125}I]ET-1- and [^{125}I]Sf6b- binding in atrium and in ventricle (Figure 3, Figure 4). In the coronary artery,

all three cold ligands (ET-1, [Ala^{1,3,11,15}]ET-1, and BQ-123) caused a similar amount of total displacement both for [^{125}I]ET-1 and [^{125}I]Sf6b (Figure 5). Taken together, this would indicate that the use of ET-1 to define non-specific binding is appropriate.

Endothelin receptors in the ventricle and the atrium

Endothelin has been shown to cause positive inotropic and / or chronotropic effects in the ventricle and atrium of several animal species [6, 8, 23] and man [5, 24, 25]. In the rabbit isolated papillary muscle, the positive inotropic response appeared to be mediated by the ET_B receptor [7], but the nature of the receptor involved in endothelin-induced rat myocyte hypertrophy and secretion of natriuretic peptide from rat myocytes [11] thus far remains unknown. The human ventricle has recently been shown to contain both ET_A and ET_B receptors, e.g. by using BQ-123 and BQ-3020 (an ET_B receptor selective compound) to displace [^{125}I]ET-1-labelled sites and also by showing the presence of mRNA strands for both the ET_A and the ET_B receptor [17]. These authors also found a (free wall) ventricular population of both ET_A and ET_B receptors in an approximate percentage of 60 and 40 percent, respectively (present study 53 and 47 percent, respectively). In our study the pIC_{50} values of ET-1, [Ala^{1,3,11,15}]ET-1 and BQ-123 also fitted with a mixed ET_A / ET_B receptor population. The high affinity of BQ-123 ($pK_D \approx 9$), presumably for the ET_A receptor, was however clearly higher in the study by Molenaar and colleagues [17] than in our study ($pIC_{50} \approx 7$). Other ET_A receptor assays, however, have reported pIC_{50} values for BQ-123 of approximately 7 [15] or 8 [20].

Also in the atrium we observed a mixed receptor population that appeared to consist of both ET_A and ET_B receptors. In the right atrium, the proportion of ET_A receptors (68% ET_A vs. 32% ET_B receptors) was somewhat higher than in the ventricle. This was possibly due to a higher density of atrial ET_A receptors and similar density of ET_B receptors, since the overal ET receptor density was higher in the atrium. Endothelin has previously been shown to elicit positive inotropic responses in the human isolated atrium [24, 25]. By contrast, ET-1 did not cause positive inotropic affects in six strips obtained from the human ventricle, that responded normally to (-)-isoprenaline [24] and caused only very small inotropic effects in another study [5]. In this respect, it is of interest that the receptor density in the ventricle of several animal species was previously found to correspond to the effectiveness of ET-1 in producing a positive inotropic effect [7]. Functionally differential effects on human atrial and ventricular tissue were also observed for 5-hydroxytryptamine, which produced a positive inotropic effect on the isolated atrium [26], that could not be shown in the ventricle [27-29]. Therefore, one should be careful to extrapolate the positive

inotropic response, elicited by endothelin in the rabbit papillary muscle [7] and in the human atrium [24, 25], to the human ventricle until further functional experiments in the human ventricle are performed.

Endothelin receptors in the coronary artery

Endothelin was originally described as a vasoconstrictor peptide [1]. Since then, various reports have appeared on the nature of the receptor mediating contraction. An ET_A receptor appeared to be involved in the porcine coronary artery contractile response to ET-1 [30], but a non-ET_A, non-ET_B receptor seemed to be involved as well [31]. This contention was based on the observation that ET-3 recognized a sarafotoxin S6c sensitive receptor (sarafotoxin S6c is ET_B receptor selective [32]), which was not recognized by ET-1, thus ruling out the ET_B receptor. ET-1 was also shown to cause potent contractions of the human isolated coronary artery [24, 33, 34], but until now the nature of this particular endothelin receptor has remained unknown.

The present results indicate that a receptor is present in the human coronary artery, which showed similar affinities for ET-1, [$Ala^{1,3,11,15}$]ET-1 and BQ-123, when compared to one of the two endothelin receptors in the human ventricle and atrium. Using in situ-hybridization to show the presence of mRNA for the receptor, Molenaar and colleagues provided further evidence that this endothelin receptor in the human ventricle and atrium is of the ET_A receptor type [17]. Considering the biphasic and shallow displacement curves (Figure 5), [^{125}I]Sf6b also appeared to label another binding site with high affinity for both [$Ala^{1,3,11,15}$]ET-1 and BQ-123 (Table 3). Apparently, this binding site does not relate to any of the thus far discovered endothelin receptors. Interestingly, unlabelled ET-1 completely displaced [^{125}I]Sf6b binding with high affinity, and thus also appeared to have affinity for this 'unknown' binding site. On the other hand, *iodinated* ET-1 did not bind to the 'unknown' binding site since ET-1, BQ-123 and [$Ala^{1,3,11,15}$]ET-1 all caused monophasic displacement of [^{125}I]ET-1. Whether indeed this [^{125}I]Sf6b-labelled binding site - with high affinity for both BQ-123 and [$Ala^{1,3,11,15}$]ET-1 - constitutes a new endothelin receptor in blood vessels, requires further investigation using other endothelin isopeptides like ET-3 and sarafotoxin S6c, in radioligand assays as well as in functional experiments. Also the existence of different affinity states for the same receptor, labelled by [^{125}I]Sf6b, but not by [^{125}I]ET-1, cannot be entirely excluded. This does however not apply to differences between high and low affinity phases for [$Ala^{1,3,11,15}$]ET-1 and BQ-123 in the atrium and ventricle, since the differences of pIC_{50} values appear too high for different affinity states of the same receptor (Table 1, Table 2). On the other hand,

vascular receptor heterogeneity was previously suggested by Sumner and co-workers, who observed that ET-3-induced contractions of the rabbit thoracic aorta were antagonized by BQ-123 with an estimated pK_B value of 8.3, whereas BQ-123 antagonized ET-1-induced contractile responses of the same tissue with a (presumably ET_A receptor-related) pA_2 of 6.9 [35].

Endothelin receptors in the diseased heart

When compared to healthy cardiac tissue (Bio Implant Services / Eurotransplant organ donor tissue), we observed no differences in atrial and ventricular endothelin receptor characteristics in diseased hearts (dilating cardiomyopathy, obtained from heart transplant recipients). However, it must be kept in mind that the numbers of diseased hearts investigated (n=2) is too small to note possible subtle differences. Other investigators also failed to show a difference between healthy and diseased tissue in man (e.g. Eisenmenger's syndrome, ischemic heart disease [17]). However, an experimentally induced congestive heart failure in dogs, was found to result in a downregulation of cardiac endothelin receptors, possibly due to an increased plasma endothelin level [36]. An increased plasma endothelin level has also been noticed in a variety of human cardiovascular (and non-cardiovascular) diseases in man [37].

In the human coronary artery obtained from cardiac transplantation patients for ischemic heart disease (n=2), we have neither observed a higher density of receptors nor different receptor characteristics. However, it must be stressed that the pathological changes in coronary artery diseased hearts, obtained after transplantation, are the end-stage of a disease, present in the vasculature of many subjects who are generally considered healthy.

In summary, we observed that both [^{125}I]ET-1 and [^{125}I]Sf6b labelled a population of ET_A and ET_B binding sites in the human ventricle and atrium. Using [^{125}I]ET-1 we found a single receptor population in the human coronary artery with similar characteristics as the ET_A receptor in the ventricle and atrium. However, the radioligand [^{125}I]Sf6b also labelled a binding site with characteristics different from the atrial and ventricular endothelin receptors. Further experiments are needed to demonstrate whether this binding site represents a new endothelin receptor in the human coronary artery.

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Chapter 8

Heterogeneity of endothelin receptors mediating contraction of the human isolated saphenous vein*

Summary - We investigated the effect of the endothelin$_A$ (ET_A) receptor antagonist BQ-123 (0.1 and 1 μM) on contraction of the human isolated saphenous vein induced by endothelin-1 (ET-1) or sarafotoxin S6b. Contraction to ET-1 was not affected by BQ-123. In contrast, BQ-123 biphasically attenuated contractions to sarafotoxin S6b. These data indicate that (i) ET-1 induces contractions of the human saphenous vein via a BQ-123-insensitive receptor and (ii) contractions to sarafotoxin S6b are mediated in part via a receptor, different from the receptor mediating contraction to ET-1.

8.1 Introduction

The cloning of two endothelin receptors, denoted ET_A and ET_B [1, 2], has encouraged the characterization of endothelin receptors that mediate contraction in the vascular bed of several species (e.g. Ref. 3). This was facilitated by the introduction of compounds with selectivity for one of these two receptors. BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]), for instance, has extensively been used as a selective antagonist with high affinity for the ET_A receptor [4, 5]. Although the ET_A receptor was originally believed to be the most important in mediating vascular smooth muscle vasoconstriction [6], several investigators concluded that the ET_B receptor [3], or a non-ET_A, non-ET_B receptor [7] also play a role in vasoconstriction. For this reason, and because little information is available on human vascular tissues, we investigated the effect of BQ-123 (0.1 and 1 μM) on contractions of the human saphenous vein induced by endothelin-1 (ET-1) or sarafotoxin S6b.

*, *Based on:* Bax, W.A., Bos, E. and Saxena, P.R. (1993) Heterogeneity of endothelin / sarafotoxin receptors mediating contraction of the human isolated saphenous vein. *Eur. J. Pharmacol.* **239**, 267-268.

8.2 Materials and methods

Leftover human saphenous vein was obtained intraoperatively from 9 patients (8 males, 1 female; age: 53 to 75 years), undergoing coronary bypass surgery. The tissue was immediately placed in a cold, oxygenated Krebs' bicarbonate solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_2$, and 8.3 mM glucose; pH 7.4. The vein was cleaned of adhesive fat and connective tissue, and the endothelium was removed, using a cotton swab. Ring segments (4 mm) were suspended in 15 ml organ baths to measure isometric tension. The absence of the endothelium was verified by observing lack of relaxation to acetylcholine (1 μM) or bradykinin (1 μM) after precontraction with noradrenaline (0.1 μM). After measuring the contractile response to K^+ (100 mM), the segments were either incubated with BQ-123 (0.1 or 1 μM) for 30 min, or remained untreated as a control segment. Subsequently, a concentration response curve to ET-1 or sarafotoxin S6b was obtained in all vessel segments. Contraction was expressed as a percentage of K^+ (100 mM)-induced contractions.

ET-1, sarafotoxin S6b and BQ-123 were purchased from Neosystem S.A., Strasbourg, France; all other drugs from Sigma Chemical Co., St. Louis, USA.

8.3 Results

Both ET-1 and sarafotoxin S6b caused concentration dependent contractile responses of the vessel segments (pD_2: 8.06 ± 0.10 and 8.13 ± 0.05, respectively; E_{MAX}: 123 ± 7% and 135 ± 6% of K^+ (100 mM)-induced contractions, respectively; n=7-9). BQ-123 (0.1 and 1 μM) did not affect ET-1-induced contraction. The contractile responses, induced by sarafotoxin S6b in concentrations not higher than 1 nM, also remained unaffected by BQ-123. However, contractions induced by higher concentrations of sarafotoxin S6b were concentration-dependently attenuated by BQ-123 (0.1 and 1 μM; Figure 1).

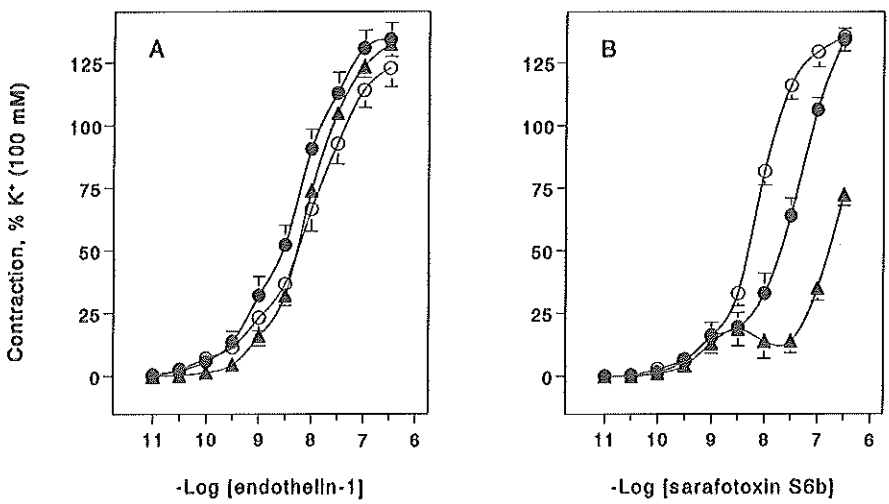

Figure 1. Contractions of the human isolated saphenous vein to endothelin-1 (panel A) or sarafotoxin S6b (panel B), without (○, control) or in the presence of BQ-123 (0.1 μM, ●; 1 μM, ▲). The concentration refers to mol/l. Contractions are expressed as a percentage of K^+ (100 mM)-induced contractions. n=7-9.

8.4 Discussion

We conclude that ET-1 contracts the human isolated saphenous vein via a BQ-123-insensitive receptor. Since BQ-123 has high affinity for ET_A receptors [4, 5] it appears unlikely that ET_A receptors are involved. On the other hand, BQ-123 caused a biphasic inhibition of sarafotoxin S6b-induced contractions, indicating heterogeneity of the receptor population mediating the contractile response to sarafotoxin S6b. It appears reasonable to assume that the BQ-123-insensitive receptor, which mediates the contractile response to low concentrations of sarafotoxin S6b, is similar to the receptor that mediates the contractile response to ET-1. The exact nature of this receptor remains to be determined. The BQ-123-sensitive receptor, which mediates the contractile response to higher concentrations of sarafotoxin S6b, appears to be different from the ET_A receptor;

since ET-1 has higher affinity than sarafotoxin S6b for the ET_A receptor [1], one would expect that the contractile response to ET-1 would also be attenuated by BQ-123. Therefore, the present data indicate that BQ-123 may have relatively high affinity for a non-ET_A, non-ET_B receptor. Interestingly, a previous study using quantitative receptor autoradiography in the human coronary artery, also indicated that [^{125}I]sarafotoxin S6b but not [^{125}I]endothelin-1 labeled a non-ET_A, non-ET_B receptor with high affinity for BQ-123 [8].

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Chapter 9

Different endothelin receptors involved in endothelin-1- and sarafotoxin S6b-induced contractions of the human isolated coronary artery

Summary - Endothelin receptors, that mediate contraction of the human isolated coronary artery, were characterized using a number of agonists and antagonists. Contraction induced by the non-selective agonists endothelin(ET)-1 and sarafotoxin S6b, was compared in endothelium-intact and endothelium-denuded ring segments. The effects of ET-1 and BQ-123 (an ET_A receptor antagonist) were investigated both in ring segments and in spirally cut strips. Lastly, the effect of phosphoramidon was studied on contraction induced by big-ET-1.

The order of agonist potency (pD_2) in endothelium-intact coronary artery ring segments was: ET-1 (8.27) ≈ sarafotoxin S6b (8.16) > big-ET-1 (< 7.1) ≈ ET-3 (< 6.9). [$Ala^{1,3,11,15}$]ET-1 (ET_B receptor agonist) caused significant contraction at 1 μM only, whereas 0.3 μM big-ET-3 had no effect. Removal of the endothelium in ring segments did not affect the contractile response to ET-1 or to sarafotoxin S6b. After a full concentration response curve with ET-1 or sarafotoxin S6b, further contractions of the endothelium-intact coronary artery segments could only be achieved by applying ET-1 in segments exposed to sarafotoxin S6b, and not the reverse.

0.1 μM BQ-123 antagonized contractions of endothelium-intact ring segments induced by sarafotoxin S6b (pK_B: 7.86). Only 10 μM BQ-123 antagonized contractions induced by ET-1 (pK_B: 5.75). Also FR139317 was more potent against sarafotoxin S6b (pK_B: 8.24-8.47) than against ET-1 (pK_B: 6.11). [$Ala^{1,3,11,15}$]ET-1 (1 μM) had no effect on the contractile response to ET-1 or to sarafotoxin S6b.

*, *Based on:* Bax, W.A., Aghai, Z., Van Tricht, C.L.J., Wassenaar, C. and Saxena, P.R. (1994) Different endothelin receptors involved in endothelin-1- and sarafotoxin S6b-induced contractions of the human isolated coronary artery. *Br. J. Pharmacol.* **113**, 1471-1479.

In strip preparations with intact endothelium, the pD_2 of ET-1 increased to 9.04 ± 0.16 (vs. 8.50 ± 0.07 in rings), and 1 μM BQ-123 caused a rightward shift of the ET-1-induced concentration response curve (pK_B: 6.62 vs. 5.75 in rings).

Contractile responses to big-ET-1 of endothelium-intact coronary artery segments were attenuated in the presence of 100 μM phosphoramidon, indicating conversion of big-ET-1 to ET-1 within the coronary artery segment.

The present study indicates that ET-1 and sarafotoxin S6b contract the human isolated coronary artery via different receptors, which can probably be best characterized as subtypes of the ET_A receptor. Furthermore, it is demonstrated that the type of preparation (ring or strip) may affect the potency of ET-1 as an agonist, and of BQ-123 as an antagonist.

9.1 Introduction

The cloning of endothelin ET_A [1] and ET_B [2] receptors, and the subsequent development of relatively selective receptor agonists and antagonists, have stimulated the characterization of functional receptors, mediating vascular smooth muscle contraction to endothelin isopeptides. For instance, ET_A receptors have been shown to mediate contractile responses in the rat aorta [3], whereas ET_B receptors were found to mediate contraction of the rabbit saphenous vein [4]. Other smooth muscle preparations were found to contract via a mixed receptor population, or via non-ET_A, non-ET_B receptors (e.g. the pig coronary artery [5, 6]. In particular, the putative ET_A receptor antagonist BQ-123 (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]) [7] was found to discriminate between contractions induced by endothelin(ET)-1 and contractions induced by endothelin(ET)-3 or sarafotoxin S6b in rat aorta [3], rat vas deferens [8, 9] and goat cerebral artery [10]. Also in the human saphenous vein it was found that BQ-123 antagonized sarafotoxin S6b-induced contractions with higher affinity than ET-1-induced contractions [11]. Similar data were obtained in the human umbilical artery [12]. These apparently heterogeneous receptor populations could not be readily characterized as 'typical' ET_A or ET_B receptors.

In the human isolated coronary artery, Godfraind [13] observed differential effects of BQ-123 against ET-1- and ET-3-induced contractile responses. Furthermore, the nature of the contractile endothelin receptors varied between proximal and distal coronary artery segments; distal segments were more sensitive to both the agonist effect of ET-1 (as was also observed by Chester and co-workers [14]) and the antagonist effect of BQ-123. It was

therefore concluded that distal parts of the artery contract via ET_A receptors only, but that other receptors are involved in the contractile response of proximal segments [13]. Using quantitative receptor autoradiography in the human coronary artery, it was previously shown that [^{125}I]ET-1 labelled a homogeneous receptor population in the media of proximal segments of the human coronary artery. [^{125}I]Sarafotoxin S6b labelled a similar receptor, but also labelled a non-ET_A, non-ET_B receptor with relatively high affinity for BQ-123 and [Ala^{1,3,11,15}]ET-1 [16] (ET_B receptor selective [15]).

In the present study, we further characterized endothelin receptors that mediate contraction of the human isolated coronary artery, using BQ-123 and FR139317 (ET_A receptor selective [17]) as antagonists. We also examined whether the response to ET-1 or to sarafotoxin S6b is altered in the presence or absence of functionally intact endothelium [18]. We hypothesized that part of the previously found differences between large proximal and small distal coronary artery segments [13, 14], could be due to facilitated diffusion of the large peptide molecules to the receptors in distal coronary artery segments, rather than to a different receptor profile. To verify this hypothesis we compared the effect of ET-1 and BQ-123 on ring segments and on spirally cut coronary artery strips. Lastly, we investigated whether local conversion of big-ET-1 to ET-1 [19, 20] is involved in the contractile response to big-ET-1. A part of this study was presented to the joint meeting of the British Pharmacological Society and the Società Italiana Farmacologia [21].

9.2 Methods

Tissue preparation and experimental procedure

The right epicardial coronary artery was obtained from 43 hearts of organ donors, who had died of non-cardiac disorders less than 24 hours before the tissue was taken to the laboratory (25 cerebrovascular accident, 16 polytrauma, 2 cerebral hypoxia; 24 male, 19 female; age 1-54 years). The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services Foundation / Eurotransplant Foundation) after removal of the aortic and pulmonary valves for homograft valve transplantation. The study was approved by the Joint Medical Ethics Committee of the Erasmus University Rotterdam and the University Hospital Rotterdam 'Dijkzigt'. The hearts were stored at 0-4 °C in commonly used sterile organ protecting solutions (University of Wisconsin (UW) solution, HTK-Bretschneider solution, or EuroCollins solution, see Ref. 22), immediately following circulatory arrest. After arrival in the laboratory, the right coronary artery was removed and placed in a cold,

oxygenated Krebs bicarbonate solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_2$ and 8.3 mM glucose; pH 7.4. Segments 2 (mid segment) and 3 (distal segment) [23] of the coronary artery were cut into rings with similar diameter and approximately 4 mm of length. The rings were suspended on stainless steel hooks in 15 ml organ baths containing the Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37° C. Vessel segments containing macroscopically visible atherosclerotic lesions were not used. In some ring segments, the endothelium was removed, using a cotton swab and a watchmakers' forceps. However, if not mentioned otherwise, results refer to ring segments with intact endothelium. For the comparison of ring and strip segments, we made two such pairs from adjacent portions of the coronary artery with intact endothelium. The 4 mm rings were prepared as described above, while the strips were cut in a spiral manner (diameter 1 mm) and attached to silk threads.

All segments were allowed to equilibrate for at least thirty min and were washed every fifteen min. Changes in tension were recorded using a Harvard isometric transducer. Preparations were stretched to a stable tension of approximately 20 mN. The tissue was exposed to K^+ (30 mM) twice. Subsequently, the functional integrity of the endothelium was verified by observing relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). After washout, the tissue was exposed to 100 mM K^+ to determine the maximal contractile response to K^+, which was expressed as mN. The tissue was allowed to equilibrate in the Krebs solution for a period of thirty min.

Determination of agonist and antagonist potency

After this period of equilibration, some segments were treated with vehicle only (controls), whereas others were incubated with an antagonist for thirty min. After that, a cumulative concentration response curve was obtained on every segment. Responses were expressed as a percentage of the contractile response to 100 mM K^+. Curves obtained in the presence of a receptor antagonist were compared to the control curves. All curves were obtained in a paired, parallel experimental set-up. Some concentration response curves were obtained in duplicate or triplicate. These curves were averaged and considered as a single curve in further analysis.

Analysis of Data

Curves were analyzed by means of a computerized curve fitting technique [24] to obtain E_{MAX} (maximal response), and pD_2 (-Log of the molar concentration of an agonist needed to reach half of its maximal effect, i.e. $-LogEC_{50}$) values, which were averaged for the respective agonists. For antagonists against ET-1 or sarafotoxin S6b, a pK_B value was calculated, assuming the nature of antagonism to be competitive, using the equation described by Furchgott [25]:

$$pK_B = -Log[B] + Log\{([A_2]/[A_1])-1\},$$

where [B] is the antagonist concentration and $[A_1]$ and $[A_2]$ are, respectively, the EC_{50} values of the agonist in the absence and presence of the antagonist. For calculation of pK_B values, only paired experiments were taken into account. pK_B values are given only when a more than 2-fold shift of the EC_{50} was obtained. All data are presented as mean ± s.e.mean. pD_2 and E_{MAX} of agonist-induced contractions without or in the presence of an antagonist were compared using a paired Student's *t*-test. Mean contractile responses were compared using an Analysis of Variance (ANOVA) followed by a Student's *t*-test, for paired data where appropriate. A correlation coefficient, *r*, was calculated according to Pearson. A *P* value less than 0.05 was assumed to denote a significant difference.

Compounds

The compounds used in this study were purchased from the sources indicated: Prostaglandin $F_{2\alpha}$ (tris salt), substance P acetate and phosphoramidon: Sigma Chemical Co. (St. Louis, USA); ET-1: Saxon Biochemicals GMBH (Hannover, Federal Republic of Germany); ET-1, sarafotoxin S6b, FR139317 and BQ-123 (sodium salt): Neosystem S.A. (Strasbourg, France); sarafotoxin S6b, ET-3, big-ET-1 and BQ-123 (sodium salt): Novabiochem AG (Läufelfingen, Switzerland); sarafotoxin S6b, big-ET-3, and $[Ala^{1,3,11,15}]$ET-1: Peninsula Laboratories (Belmont, USA). It should be noted that due to non-availability of the compounds and/or cost factors, we had to order ET-1, sarafotoxin S6b, and BQ-123 from more than one source. Although direct comparisons were not made, the compounds obtained from different sources did not differ in potency. Stock solutions for $[Ala^{1,3,11,15}]$ET-1, and ET-1 (Saxon) were prepared in 0.1% acetic acid. Other compounds were dissolved in water.

9.3 Results

Basic properties of the preparations

All ring segments, where endothelium was left intact, relaxed to 1 nM substance P after precontraction to 1 μM $PGF_{2\alpha}$ (73 ± 5% of the contractile response to $PGF_{2\alpha}$; n=43). This is in accordance with previous findings with substance P in the human isolated coronary artery obtained from patients undergoing cardiac transplantation [26]. 100 mM K^+ caused a mean contractile response of 45 ± 3 mN (n=43). Where the endothelium had been removed from the ring segments, the relaxant response to substance P was virtually abolished (2 ± 1% of the contractile response to 1 μM $PGF_{2\alpha}$; n=13), and the contractile response to potassium was decreased from 37 ± 5 mN to 21 ± 3 mN (n=13; $P<0.01$). In strip preparations (n=5), the relaxant response to substance P was 31 ± 12% of the contractile response to 1 μM $PGF_{2\alpha}$, and the contractile response to 100 mM K^+ was 20 ± 6 mN.

Effect of agonist peptides

In endothelium-intact coronary artery segments, sarafotoxin S6b, ET-1, big-ET-1, and ET-3 caused concentration dependent contractions. [$Ala^{1,3,11,15}$]ET-1 caused a small contractile response at the highest concentration used (1 μM), whereas big-ET-3 had no effect at 0.3 μM (Figure 1). For big-ET-1, ET-3 and [$Ala^{1,3,11,15}$]ET-1 the highest concentration tested (1 μM) may not have reached the maximal contractile response, which could affect the given approximate pD_2 and E_{MAX} values. The order of potency of the agonists tested was: ET-1 ≈ sarafotoxin S6b > big-ET-1 ≈ ET-3 > [$Ala^{1,3,11,15}$]ET-1 ≥ big-ET-3 (for E_{MAX} and pD_2, see Table 1). The contractile response to ET-1 or sarafotoxin S6b in ring segments was not affected by removing the endothelium (Figure 2). Comparison of the E_{MAX} of 21 paired vessel segments, in which a concentration response curve with both ET-1 and sarafotoxin S6b was constructed, revealed that the E_{MAX} but not the pD_2 of sarafotoxin is slightly but significantly different from that of ET-1 (105.7 ± 4.9% vs. 95.2 ± 3.9%, respectively, $P=0.01$). No age-related variation was observed for the pD_2 of ET-1 (n=37) and sarafotoxin S6b (n=26) (correlation age - pD_2: $r=-0.12$ and -0.05, $P=0.24$ and 0.40, respectively). Only the E_{MAX} of ET-1, but not of sarafotoxin S6b, was found to be somewhat higher in younger patients (correlation age - E_{MAX}, ET-1: $r=-0.36$, $P=0.02$; sarafotoxin S6b: $r=0.07$, $P=0.37$).

Table 1. Potency (pD_2 (-Log [EC_{50}, mol/l])) and maximal effect (E_{MAX}, expressed as a percentage of the contractile response to 100 mM K^+) of endothelin receptor agonists.

Agonist	Concentration range studied	n	pD_2	E_{MAX}
ET-1	30 pM - 0.3 μM	37	8.27 ± 0.06	102.7 ± 5.4%
Sarafotoxin S6b	30 pM - 0.3 μM	26	8.16 ± 0.05	104.5 ± 5.4%
Big-ET-1	0.1 nM - 1 μM	4	<7.1 ± 0.02[1]	>124.3 ± 12.4%[1]
ET-3	0.1 nM - 1 μM	4	< 6.9 ± 0.1[1]	>84.4 ± 9.6%[1]
[$Ala^{1,3,11,15}$]ET-1	0.1 nM - 1 μM	7	n.c.[1]	>11.4 ± 3.6%[1]
Big-ET-3	0.1 nM - 0.3 μM	3	No Effect	No Effect

n, Number of segments tested;
n.c., Not calculated;
[1], Maximal effect possibly not reached at 1 μM.

Figure 3 shows experimental recordings of responses obtained with ET-1 and sarafotoxin S6b in endothelium-intact coronary artery segments. The addition of 0.1 μM sarafotoxin S6b at the end of a full concentration response curve with ET-1 did not result in a further increase of tension (Figure 3A). However, in a parallel vessel segment, the addition of 0.1 μM ET-1 after a full concentration response curve with sarafotoxin S6b, caused a further increase of tension (Figure 3B and also 3D). Figure 3C shows that the maximal contractile response to sarafotoxin S6b (but also to ET-1: not shown) slowly faded after the highest concentration of agonist had been added. The mean contraction (as a percentage of contraction induced by 100 mM K^+), directly and sixty min after adding 0.3 μM ET-1 or 0.3 μM sarafotoxin S6b was: 84.0 ± 5.2% and 26.2 ± 3.5%, respectively, for ET-1 (n=18 segments from 10 hearts), and 90.7 ± 10.0% and 56.3 ± 11.8%, respectively, for sarafotoxin S6b (n=10 segments from 5 hearts). Sixty min after the maximal concentration of sarafotoxin S6b had been added (not shown in Figure 3), the subsequent addition of 0.03 μM ET-1 resulted in an increase of contractile force (from 33.0 ± 11.7% to 81.1 ± 6.0% of contraction induced by 100 mM K^+, n=5), whereas the subsequent adding of 0.03 μM sarafotoxin S6b had no further effect (from 66.2 ± 3.2% to 64.3 ± 4.0% of contraction induced by 100 mM K^+, n=5). When 0.1 μM ET-1 was

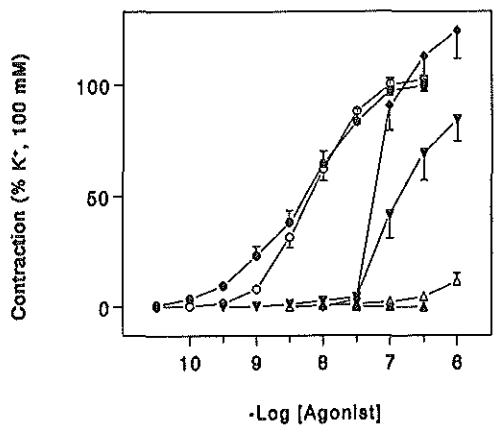

Figure 1. Contractions of the endothelium-intact human isolated coronary artery to endothelin receptor agonists. ●, ET-1 (n=37); ○, sarafotoxin S6b (n=26); ♦, big-ET-1 (n=4); ▼, ET-3 (n=4); ∆, [Ala^{1,3,11,15}]ET-1 (n=7); ▲, big-ET-3 (n=3). Agonist concentration refers to mol/l. pD_2 and E_{MAX} are mentioned in Table 1. Where no error bar is visible, it falls within the limits of the symbol.

added after this failure of 0.03 μM sarafotoxin S6b to produce further contraction, an increase of contractile force to 99.6 ± 3.1% of contraction induced by 100 mM K^+ was observed (n=5; $P<0.01$). Sixty min after the maximal concentration of ET-1 had been added, subsequent addition of neither 0.03 μM ET-1 nor 0.03 μM sarafotoxin S6b resulted in a further increase of contraction (from 22.1 ± 4.9% to 22.6 ± 5.0% (n=10) and 31.3 ± 6.2% to 32.1 ± 6.1% (n=8) of contraction induced by 100 mM K^+, respectively).

Antagonist effects

In endothelium-intact coronary artery segments 0.1 μM BQ-123 antagonized contractions induced by sarafotoxin S6b resulting in a pK_B of 7.86 ± 0.08 (n=6). This same concentration of BQ-123 caused a small increase of contraction induced by ET-1 (n=7). Only 10 μM BQ-123 antagonized contractions induced by ET-1 (pK_B: 5.75 ± 0.18; n=4). FR139317 was also more potent against sarafotoxin S6b than against ET-1. This resulted in pK_B values for FR139317 against sarafotoxin S6b of 8.47 ± 0.17 (3 nM) and

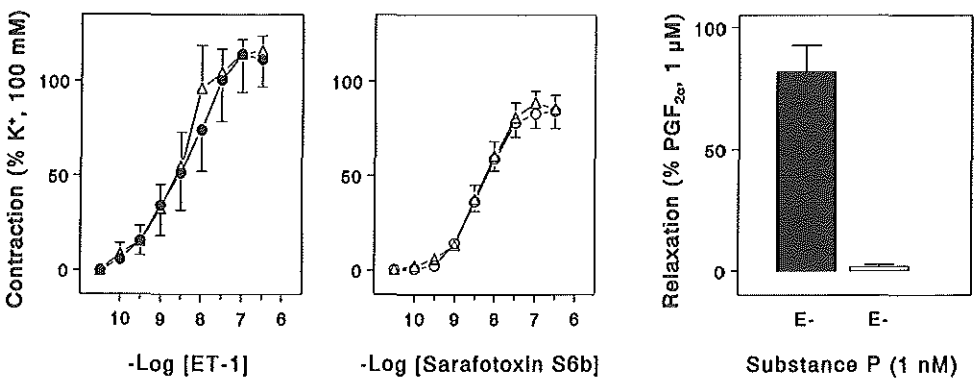

Figure 2. Left panel: Contractions of the human isolated coronary artery to ET-1 in endothelium intact (●), or endothelium denuded (▵) ring segments (n=8). Middle panel: Contractions of the human isolated coronary artery to sarafotoxin S6b in endothelium intact (○), or endothelium denuded (▵) ring segments (n=5). The concentration of ET-1 and sarafotoxin S6b refers to mol/l. Right panel: Relaxant responses to 1 nM substance P in the same segments expressed as a percentage of precontraction with 1 μM $PGF_{2\alpha}$. E+, endothelium intact; E-, endothelium denuded. Where no error bar is visible, error falls within the limits of the symbol.

8.24 ± 0.08 (0.3 μM) (both n=5). By contrast, 3 μM FR139317 applied against ET-1-induced contractions resulted in a pK_B of 6.11 ± 0.16 (n=6). The small contractile response induced by 1 μM [$Ala^{1,3,11,15}$]ET-1 faded during the thirty min incubation when tested as an antagonist. [$Ala^{1,3,11,15}$]ET-1 (1 μM) did not affect the contractile response to ET-1 or to sarafotoxin S6b (n=6) (Figure 4).

Comparison with strip preparations

ET-1 was more potent as an agonist on endothelium-intact strip preparations than on endothelium-intact ring segments (pD_2: 9.04 ± 0.16 in strips vs. 8.50 ± 0.07 in rings; n=5). The pK_B of BQ-123 (1 μM) against ET-1-induced contractions of strips was 6.62 ± 0.18 (Figure 5) vs. 5.75 ± 0.18 in rings (10 μM BQ-123).

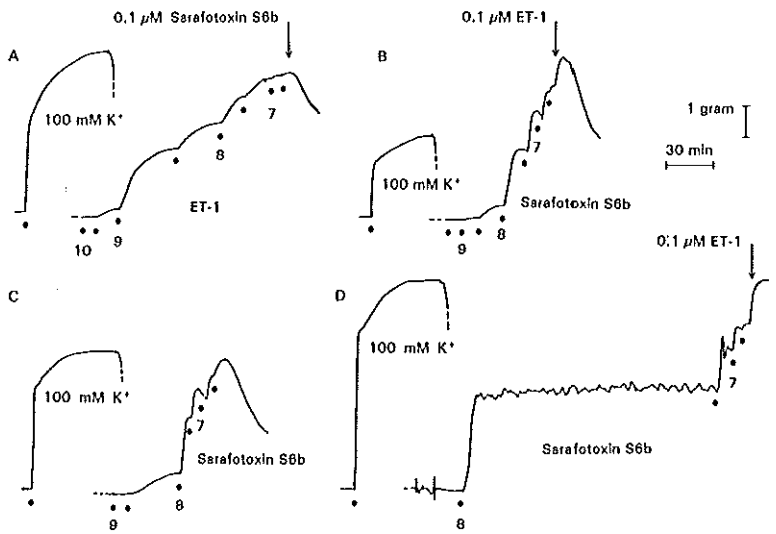

Figure 3. Experimental recordings of concentration response curves, preceded by the reference contractile response to 100 mM K^+, in four endothelium-intact coronary artery segments obtained from a 41 year old male (A and B) and a 25 year old female (C and D). **A.** After reaching the E_{MAX} in a concentration response curve with ET-1, the addition of 0.1 μM sarafotoxin S6b (arrow) does not cause an additional response. **B.** After reaching the E_{MAX} in a concentration response curve with sarafotoxin S6b, the addition of 0.1 μM ET-1 (arrow) does cause an additional contractile response. **C.** After reaching the E_{MAX} of a concentration response curve to sarafotoxin (but also to ET-1: not shown) contraction slowly faded away (also A and B). **D.** Addition of a concentration sarafotoxin S6b, causing a submaximal effect in a parallel segment, resulted in a sustained contractile response. After subsequently completing the concentration response curve in this particular segment, a further contractile response could be induced by adding 0.1 μM ET-1 (arrow).

Big-ET-1 and phosphoramidon

The concentration response curve to big-ET-1 was remarkably steep in comparison with the other endothelin isopeptides (Figure 1). As shown in Figure 6, the pretreatment of the vessel segment with phosphoramidon (100 μM) resulted in significant attenuation of the contractile responses, and in a non-parallel shift of the big ET-1-induced concentration response curve.

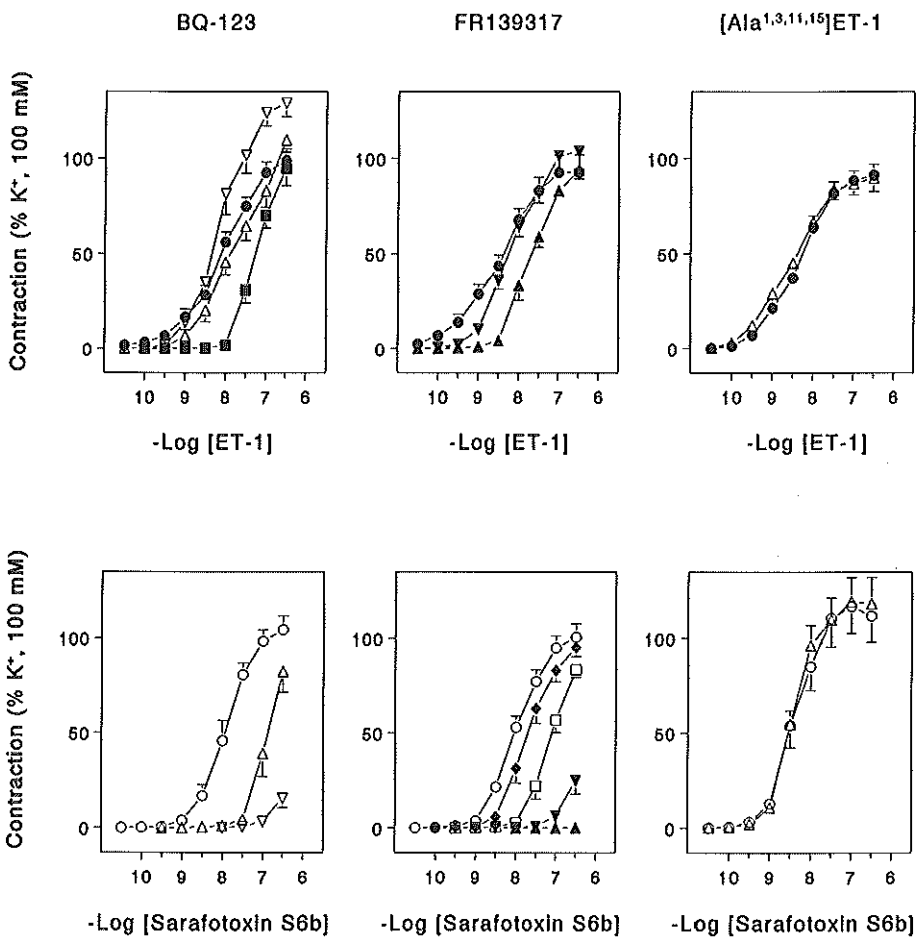

Figure 4. Contractions of the endothelium-intact human isolated coronary artery to ET-1 (top panels), and sarafotoxin S6b (bottom panels) in the absence (control ET-1: ●; control sarafotoxin S6b: ○) or presence of the endothelin receptor antagonist BQ-123 (n=4-13), FR139317 (n=5-6), or [Ala^{1,3,11,15}]ET-1 (n=6). Concentrations of receptor antagonists were 3 nM (♦), 0.03 μM (□), 0.1 μM (▽), 0.3 μM (▼), 1 μM (△), 3 μM (▲) or 10 μM (■). Concentrations of ET-1 and sarafotoxin S6b refer to mol/l. pK_B values are mentioned in the text. Where no error bar is visible, error falls within the limits of the symbol.

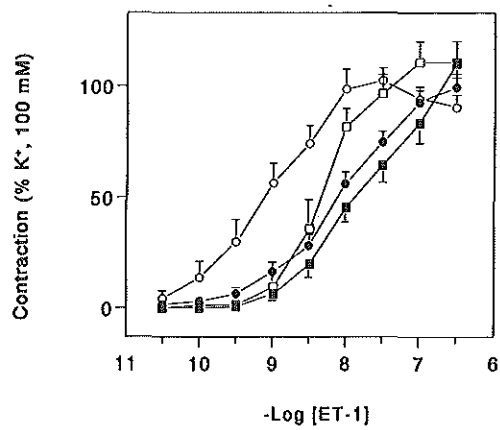

Figure 5. Contractions of the human isolated coronary artery to ET-1 in endothelium-intact ring preparations (closed symbols, n=12) and endothelium-intact spiral strip preparations (open symbols, n=5), in the absence (control, ● and ○) or presence of 1 μM BQ-123 (■ and □). For pD_2 and pK_B values, see text. The concentration of ET-1 refers to mol/l. Where no error bar is visible, error falls within the limits of the symbol.

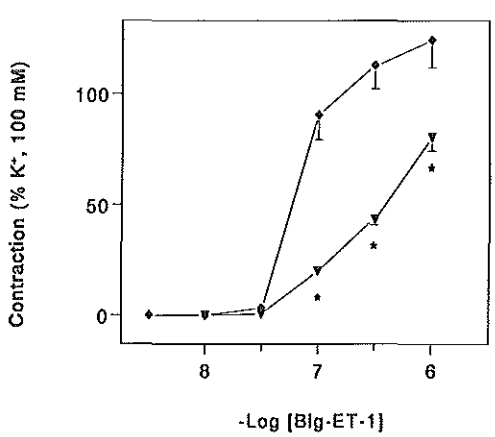

Figure 6. Contractions of the endothelium-intact human isolated coronary artery to big-ET-1 in the absence (control, ♦) and presence of 100 μM phosphoramidon (▼). n=4; *, $p < 0.05$ vs. control response. The concentration of big-ET-1 refers to mol/l. Where no error bar is visible, error falls within the limits of the symbol.

9.4 Discussion

The main findings of the present study are the observed differences between contractions induced by ET-1 and sarafotoxin S6b. The ET_A receptor antagonists BQ-123 and FR139317 were clearly more potent against sarafotoxin S6b than against ET-1. ET-1 and sarafotoxin S6b induced similar contractile responses in endothelium-intact and endothelium-denuded vessel segments. After a full concentration response curve with ET-1 or sarafotoxin S6b, further contractions of the endothelium-intact coronary artery segments could only be achieved by applying ET-1 in segments exposed to sarafotoxin S6b, and not the reverse.

It is to be noted that most concentration-response curves were obtained in vessel segments in which the endothelium was left intact, in view of the more frequent occurrence of spontaneous phasic oscillations in the absence of endothelium. These spontaneous rhythmic contractions would hinder detailed analysis of concentration response curves [27]. Destruction of the endothelium in a perfused rat mesenteric arterial bed increased the contractile response to ET-1, sarafotoxin S6b, ET-3 and big-ET-1 [28]. We established for ET-1 and sarafotoxin S6b that removal of the endothelium in coronary artery segments in our model does not alter the contractile response (Figure 2). It can however not be ruled out that contractile responses to the other agonists are modulated by the presence of endothelium.

Heterogeneity of receptors involved in the response to ET-1 and sarafotoxin S6b?

The present data supply evidence for heterogeneous mechanisms mediating the contractile response to ET-1 and sarafotoxin S6b. First, the ET_A receptor antagonists BQ-123 and FR139317 had significantly different potencies against ET-1- and sarafotoxin S6b-induced contractile responses. Secondly, after a full concentration response curve ending with 0.3 μM ET-1 or 0.3 μM sarafotoxin S6b, further contractions could only be achieved with ET-1 in segments that had been exposed to sarafotoxin S6b and not in those exposed to ET-1. Also in view of the E_{MAX} of sarafotoxin S6b being slightly, but significantly, higher than that of ET-1, it is difficult to explain why ET-1, but not sarafotoxin S6b, caused further contraction of segments exposed to the other compound. Possibly, ET-1 caused desensitization of events beyond activation of the receptor for sarafotoxin S6b, without affecting this receptor itself. The latter was also suggested by Cardell and co-workers [29], who made similar observations in the guinea-pig pulmonary artery, using ET-3 instead of sarafotoxin S6b. Hiley and colleagues [30] observed analogous cross-desensitization using ET-1 and ET-3, but suggested that ET-3 interacted with an

ET-1-insensitive accessory binding site on the receptor used by ET-1 itself. Similarly, this explanation of two agonists binding to different binding-sites on the same receptor cannot be ruled out to account for the differences in antagonist potency observed against ET-1 and sarafotoxin S6b.

To characterize endothelin receptors in the present study, several complicating factors have to be kept in mind. First, we have no knowledge of possible differential metabolic- or uptake mechanisms of any of the endothelin receptor ligands. Indeed, contractile responses to high (E_{MAX}) concentrations of ET-1 or sarafotoxin S6b (with a magnitude of over 100% of the contractile response to 100 mM K^+) fade away partially. However, this decline is apparently not due to metabolic breakdown, since in our study the contractile response to lower concentrations of the peptides was sustained (Figure 3). Secondly, one may argue that receptor pharmacodynamics are not conventionally straightforward in interactions with endothelin receptors. After activating the receptor in human and porcine smooth muscle cells, the endothelin receptor-ligand complex was found to be internalized and, thus, possibly unavailable for competition [31]. It has also been reported that an endothelin receptor antagonist like BQ-123 may act either as a competitive or non-competitive antagonist [32], depending on whether or not equilibrium conditions are present [33]. Lastly, the ligand-receptor kinetics for endothelins (e.g. different association/ dissociation rates for different endothelin isopeptides [34]) could have implications for functional studies investigating the receptors involved. On the other hand, these conceivable, but yet theoretical pitfalls should not preclude comparison of the present observations with endothelin receptor characterizations in smooth muscle preparations as reported in previous studies. Furthermore, the involvement of heterogeneous receptors is supported by the observation of an additional non-ET_A, non-ET_B binding site in human coronary arteries labelled by [^{125}I]sarafotoxin S6b, but not by [^{125}I]ET-1, with particularly high affinity for BQ-123 [16]. Also in support of the involvement of heterogeneous receptors are previous studies in human isolated saphenous veins in which BQ-123 biphasically inhibited sarafotoxin S6b-induced contractions. BQ-123 was more potent against high than against low concentrations of sarafotoxin S6b [11].

Are ET_A receptors involved in the contractile responses to ET-1 and sarafotoxin S6b?

Originally, ET_A receptors were differentiated from ET_B receptors by a high affinity of ET_A receptors for ET-1 compared to ET-3 [1, 35]. ET_B receptors, however, have similar affinity for ET-1 and ET-3 [2, 35]. Considering the difference in potency of ET-1 and ET-3 in the present study, it may be concluded that ET-1-induced responses in the present study are mediated by ET_A receptors. In addition, this order of potency virtually rules out the

involvement of the putative ET_C receptor, which has higher affinity for ET-3 than for ET-1 [36]. The presently observed agonist order of potency is similar to that in the rat thoracic aorta [3], the rabbit carotid artery [37], and the guinea-pig aorta [6]. In all of these animal preparations, it was concluded that ET_A receptors mediate the contractile response. However, the fact that in the present study BQ-123 antagonized ET-1-induced contractions only at 10 μM (pK_B = 5.75) would argue against involvement of the ET_A receptor, since BQ-123 has submicromolar [7, 35] to nanomolar [38, 39] affinity for ET_A receptors in binding studies. In our experiments 0.1 μM BQ-123, if anything, caused a small potentiation of the contractile response. The pK_B value of BQ-123 against sarafotoxin S6b appears in better agreement with ET_A receptor involvement. It is however unlikely that sarafotoxin S6b activates an ET_A receptor, which is not activated by ET-1, since ET-1 has an even higher affinity for ET_A receptors than sarafotoxin S6b [1].

The pK_B value (6.11) of FR139317 against ET-1 is in reasonable accordance with an ET_A receptor mediated mechanism. Previously found pA_2 values for FR139317 include 6.0 in the rabbit femoral artery [40], 6.65 in the guinea-pig pulmonary artery [41] and 7.2 in the human coronary artery [42] and rabbit aorta [17]. A pK_B value of FR139317 as high as 8.2-8.5 against sarafotoxin S6b-induced responses (present results) has not yet been described for an ET_A receptor-mediated functional response in organ bath experiments, and appears too high to point towards a 'typical' ET_A receptor-mediated mechanism.

Recent studies assessing the antagonist effects of BQ-123 against ET-1-induced contractions of human coronary artery rings (2 mm) report that, even at 0.1 μM, BQ-123 antagonized contractile responses to ET-1, resulting in pA_2 values of 6.4-7.5 [13, 39]. Both these studies concluded that ET_A receptors mediate the contractile responses to ET-1. It may be hypothesized that the difference of the potency of BQ-123 in those studies and our study, and also the discrepancies within other studies between large proximal and small distal coronary artery segments [13, 14], could be due to differential diffusion of the large peptide molecules to the receptors in these two types of arterial segments. Indeed we found in the present study that the type of preparation (4 mm ring or 1 mm spiral strip) made a difference in the observed potency of both ET-1 as an agonist and BQ-123 as an antagonist (Figure 5). Although the influence of the applied resting tension on strips and rings can not be excluded, the observed discrepancy may be due to different drug concentrations in the receptor compartment, caused by differences in the diffusion barrier of large molecules [43], such as the endothelins. This could play a role in the observed divergence found between proximal and distal human coronary artery segments [13, 14], possibly in addition to a different receptor profile. One may argue that partial endothelial damage of the strip preparation affected the present results. However, it must be kept in

mind that even a *complete* removal of the endothelium in ring segments did not affect the contractile response to ET-1 or sarafotoxin S6b (Figure 2). Similar diffusion-related differences may also play a role in the recently observed 3-fold leftward shift of the ET-1 concentration response curve of human vertebral arteries of infants, when compared to vertebral arteries of adults (0-2 years vs. 38-71 years) [44]. Our data in human coronary arteries do not support such age-dependent differences in the response to ET-1 or sarafotoxin S6b.

The pK_B value of 6.62 ± 0.18 for BQ-123, presently obtained in strip preparation against ET-1, would not seem out of line when compared to pA_2 values of 6.93 in the rat aorta [3], of 6.5 in the rabbit femoral artery [40], of 6.8 and 6.9 in the rabbit carotid artery and thoracic aorta, respectively [37], and even compared to pA_2 values of 6.8 - 7.5 in the more distal parts of 2 mm human coronary artery rings [13]. In all of these preparations the response was characterized as being mediated via ET_A receptors. Interestingly, Schoeffter and Randriantsoa [6], using 3 mm ring preparations, found that ET-1-induced contractile responses of the porcine coronary artery were virtually insensitive to BQ-123 (pK_B: 5.21), and concluded that these contractions were mediated by non-ET_A receptors. By contrast, an earlier study in the porcine coronary artery, applying spiral strips (diameter 1 mm), found an apparently ET_A receptor-linked pA_2 value of 7.4 [7]. Also in the porcine coronary artery, the agonist potency of ET-1 in the strip preparation [7] was clearly higher (pD_2: ≈10) than in the ring preparation studied by Schoeffter and Randriantsoa [6] (pD_2: 8.3).

The presence of mRNA encoding for ET_A receptors was demonstrated in smooth muscle cells of the human coronary artery [39, 45]. In a receptor autoradiography analysis of endothelin receptors in the human coronary artery, it was shown that 30 pM [^{125}I]-ET-1 labelled a homogeneous receptor population in the human coronary artery with similar characteristics as the ET_A receptors identified in atrium and ventricle [11]. Also Davenport and colleagues [46], studying binding displacement of 100 pM [^{125}I]-PD151242, concluded that ET_A receptors were present in the media of (atherosclerotic) human coronary artery.

Taking into account *(i)* the higher potency of ET-1 compared to ET-3, *(ii)* the presently found pK_B value (6.62 ± 0.18) for BQ-123 against ET-1 in strip preparations, *(iii)* the potency of FR139317 in coronary artery rings, and *(iv)* the previously obtained evidence from radioligand binding and molecular biology experiments for the presence of ET_A receptors in the human coronary artery, we have to conclude that contractile responses to ET-1 were most likely mediated via an ET_A receptor, despite a pK_B value of only 5.75 for BQ-123 against ET-1-induced responses in 4 mm coronary artery rings. We showed that the type of preparation can affect the potency of both ET-1 and BQ-123,

which may have significant implications for the use of these compounds in other studies involving endothelin receptor characterization. Considering the associated high pK_B values of BQ-123 and especially FR139317, we propose that contractions to sarafotoxin S6b are mediated via another subtype of ET_A receptors. A similar suggestion has been made by Salom and co-workers [10] for the effects of sarafotoxin S6b in the goat cerebral artery. Possibly, the effects of ET-3 (also extremely potently antagonized by BQ-123) are mediated via the same receptor, not only in the human isolated coronary artery [13], but also in the human small omental vein [47], in the rat vas deferens [8, 9], and in the guinea-pig left atrium [48]. Although the above mentioned complicating receptor dynamics or kinetics can not entirely be ruled out, no evidence is available for such mechanisms yet.

Are ET_B receptors involved in contractile responses?

The very small contractile response elicited by high concentrations of [$Ala^{1,3,11,15}$]ET-1 in the human isolated coronary artery would argue against the involvement of ET_B receptors. Davenport and co-workers [39] found a complete lack of agonist effect in the human coronary artery of both [$Ala^{1,3,11,15}$]ET-1 and BQ-3020, another ET_B receptor selective agonist [49]. In the pig coronary artery [$Ala^{1,3,11,15}$]ET-1 acted as a partial agonist with a pD_2 of 7.4 [6]. Furthermore, [$Ala^{1,3,11,15}$]ET-1 induced phosphoinositol turnover in human ET_B receptor-transfected COS-7 cells (K_D 20 nM [50]), and caused relaxation of the porcine pulmonary artery (ED_{40} 4.4 nM [15]). To verify whether [$Ala^{1,3,11,15}$]ET-1 acted as a partial agonist in this preparation, concentration response curves to both ET-1 and sarafotoxin S6b were constructed in the presence and absence of 1 μM [$Ala^{1,3,11,15}$]ET-1, but no antagonism was observed (Figure 4). Thus, in the present study, no evidence was obtained to support functional involvement of contractile ET_B receptors. Also in our radioligand binding study, in which either 30 pM [^{125}I]-ET-1 or [^{125}I]-sarafotoxin S6b were used, no indication was found for the presence of ET_B receptors on the smooth muscle of the human coronary artery [16]. However, in another investigation, where 100 pM [^{125}I]-ET-1 was used as a radioligand, it was concluded that a small proportion (13%) of ET_B receptors was present in the human coronary artery [39]. The presence of mRNA encoding for ET_B receptors has been shown in human coronary artery smooth muscle cells [39, 45]. Possibly, these ET_B receptors do not play a major role in endothelin-induced vasoconstriction, but they may be involved in other yet unknown effects. However, recently it was shown in the human internal mammary artery and vein, that ET-1 may induce contractions via a mixed ET_A/ET_B receptor population [51].

Effects of big-ET-1 and phosphoramidon

The 21-amino acid peptide ET-1 is formed after cleavage between Trp^{21} and Val^{22} of a 38 amino acid precursor peptide, big-ET-1 [19, 20]. The contractile response of big-ET-1 in isolated tissues may develop directly via binding of big-ET-1 to vascular smooth muscle receptors [52] as well as indirectly via ET-1 cleaved from big-ET-1 by the endothelin-converting-enzyme (ECE), which is reflected by the steep nature of the concentration response curve. The activity of ECE was found to vary between different vascular beds [53], as shown by the ability of phosphoramidon, a metalloprotease inhibitor of ECE [20], to inhibit the contractile response induced by big-ET-1. Since in the present study, pretreatment with 100 μM phosphoramidon partially reduced the contractile response to big-ET-1, we conclude that ECE, present in the coronary artery, contributed to the contractile response induced by big-ET-1. It has been suggested that ECE has substrate specificity for big-ET-1 compared to big-ET-3 [20]. Apart from this explanation, the absence of contractile effects to big-ET-3 in the present study could also result from the lower contractile potency of ET-3 compared to ET-1 (Table 1), combined with, possibly, a low affinity of big-ET-3 itself for vascular contractile receptors.

In conclusion, the present study showed that ET-1 and sarafotoxin S6b contract the human isolated coronary artery via different receptor mechanisms, which can probably be best characterized as subtypes of the ET_A receptor. Responses to ET-1 and sarafotoxin S6b are not modified by endothelial denudation. This study also showed that the type of preparation (ring or strip) may affect the potency of ET-1 as an agonist, and of BQ-123 as an antagonist. Finally, the contractile response to big-ET-1 is mediated in part by conversion to ET-1, taking place within the vascular wall of the human isolated coronary artery.

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Chapter 10

The current endothelin receptor classification: Time for reconsideration?*

Summary - The possible involvement of endothelins in a variety of diseases has attracted the attention of many pharmacologists in search of a novel therapeutic approach. The rapid development of endothelin (ET) research resulted in the molecular characterization and pharmacological recognition of ET_A and ET_B receptors, and in development of compounds selective for these receptors. However, the characterization of receptors in various assays showed that a number of effects is mediated by receptors, which do not fit the present criteria for ET_A or ET_B receptors. This review addresses endothelin receptors in general, and atypical receptors in particular.

10.1 Introduction

Endothelin (ET) was discovered and recognized as a potent vasoconstrictor peptide only 7 years ago [1]. Three distinct endogenous endothelin isoforms (ET-1, ET-2, and ET-3) are cleaved from the endothelin precursors big-ET-1, big-ET-2 and big-ET-3 by an endothelin converting enzyme. Increased concentrations of endothelins have been observed after myocardial infarction, in atherosclerosis, (pulmonary) hypertension, migraine and many other diseases (review: see Ref. 2). Although recent advances towards the elucidation of the molecular structure of endothelin converting enzymes [3] will undoubtedly be followed by the development of endothelin converting enzyme inhibitors, efforts have so far primarily been directed to the development of endothelin receptor antagonists for clinical purposes. Indeed ET_A and ET_B receptors were cloned [4, 5], and selective ligands for these receptors have been recognized.

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10.2 The current criteria for endothelin receptor classification

Receptor classification in general should be based on three criteria [6,7]: (*i*) structural (gene nucleotide and amino acid sequence of the receptor protein), (*ii*) transductional (receptor-effect coupling), and (*iii*) operational (receptor-(ant)agonist interaction). Endothelin receptors are currently classified based on the consensus view of the subcommittee of IUPHAR, primarily according to endothelin isopeptide potency order, but also according to the potency of some antagonists. In so-called type I responses, ET-1 is more potent than ET-3, whereas in type II responses both isopeptides have similar potency. In studies applying techniques to express cDNA for the currently known endothelin receptors, it was established that the type I and type II responses correspond to ET_A and ET_B receptors, respectively [8].

Gene nucleotide and amino acid sequence of the receptor protein

ET_A and ET_B receptors have approximately 63% amino acid homology. Southern blots of the human genomic DNA, using cDNA probes for the ET_A and ET_B receptor under low stringency, revealed only two signals, probably corresponding to human ET_A and ET_B receptor genes. Thus it appeared that other endothelin receptors, if existent, would probably have a considerably different amino acid sequence [9]. It should however be noted that amino acid homology may be indicative of operational and functional similarity, but that this is not a general prerequisite. 5-HT_{1B} and 5-$HT_{1D\beta}$ receptors, for instance, have a clearly different pharmacological profile for a number of compounds, despite a 96% amino acid homology in the transmembrane spanning region. In contrast, 5-$HT_{1D\beta}$ and 5-$HT_{1D\alpha}$ receptors are pharmacologically practically indistinguishable, but have a relatively moderate 77% amino acid homology in the transmembrane spanning region [7].

Recently, the identification of cDNA for a receptor in xenopus dermal melanophores was reported, with relatively high affinity for ET-3. This receptor had approximately 50% amino acid homology with ET_A and ET_B receptors [10]. It is however not yet certain whether it actually represented the putative ET_C receptor, highly selective for ET-3, which was reported in operational studies in e.g. bovine endothelial cells [11], or whether it represented the xenopus variant of, for instance, ET_B receptors. Because of the scarcity of functional correlates, and because of the lack of selective ET_C receptor ligands other than ET-3, this review will not discuss ET_C receptors in further detail.

Second messenger mechanisms

Both ET_A and ET_B receptors have been described to be coupled to phosphatidyl-inositol-biphosphate (PIP_2) hydrolysis via G-protein-coupled phospholipase C, and to the generation of inositol phosphates (IP) and diacyl-glycerol, resulting in an increased concentration of intracellular Ca^{2+} [8]. In transfected Chinese hamster ovary cells it was observed that ET_A receptors induced accumulation of cAMP, whereas ET_B receptors inhibited forskolin-stimulated cAMP production. The stimulation of adenylyl cyclase, mediated by ET_A receptors, was however less efficient than the stimulation of IP formation, which raises questions about the physiological relevance of adenylyl cyclase as a second messenger system in these cells [12].

Less is known about transduction of receptors that do not resemble the ET_A or ET_B type. Kumar and co-workers [13] observed endothelin receptors in the follicular membranes of xenopus laevis oocytes, resembling human ET_A receptors in their affinity for ET-1, ET-3 and sarafotoxin S6c, but with an atypically low affinity for BQ123. Activation of this receptor, which was considered a subtype of ET_A receptors (ET_{AX}), led to mobilization of Ca^{2+}, which was blocked by treatment that uncouples gap junctions. In contrast, Ca^{2+} mobilization induced by expressed human ET_A receptors was not sensitive to such treatment. In another study, [^{125}I]-ET-1 binding to human brain endothelial cells revealed a high and a low affinity binding site [14]. The high affinity binding site had the order of affinity: ET-1 > ET-2 > sarafotoxin S6b > ET-3, which resembled the ET_A receptor, and also matched the order of potency for IP accumulation in these cells. The order of affinity for displacement of the unidentified low affinity [^{125}I]-ET-1 binding site (sarafotoxin S6b > ET-2 > ET-1=ET-3) did not match the order of potency for IP accumulation. Other second messenger systems were not examined in the latter study.

Operational characterization of endothelin receptors

Despite the structural and transductional data with respect to endothelin receptors, the present classification relies largely on operational evidence [8], i.e. data obtained in functional or radioligand binding experiments. In addition to the frequently applied potency order of the endogenous ET-1 and ET-3, a number of synthetic compounds has been identified with selectivity for ET_A or ET_B receptors. Some of the most often applied compounds are summarized in Table 1.

The operational and functional characterization of endothelin receptors is hampered by several pitfalls. First, endothelin peptides may be internalized after binding to the receptor [15]. Therefore, agonists can become unavailable for classical ligand-receptor

Table 1. The affinity (nM) of endothelin receptor ligands for ET_A and ET_B receptors.

nM	ET_A receptors		ET_B receptors			
Ligand	K_i	IC_{50}	K_i	IC_{50}	K_D	Ref.
ET-1	3.5, 0.58, 0.92[a]	0.16, 0.29	0.95, 0.12	1.6, 0.06, 0.44	0.003	4, 9, 57-61
ET-3	1000, 83, 900[a]	5.0, 150	2.0, 0.13	1.6, 0.06, 0.11	0.014	4, 9, 57-61
Sarafotoxin S6b	52[a]					4
Sarafotoxin S6c	2800	1300	0.29	0.3, 0.12	0.24	58-61
[$Ala^{1,3,11,15}$]ET-1		398		0.25	20	59, 61
BQ123	25, 17	13, 63	31000, 11100	>10000, >100000	285	57-61
FR139317	1[a]	6.3, 13	7300[a]	20000, >100000		59, 60, 62
BQ788		1300		1.2		28
Ro462005		200, 360		160, 530		59, 60
Bosentan	6.5		343			63
SB 209670	0.4	2.0	15	32		59, 64
BMS182874	63	1600	55000	>10000		59, 65
97-139	1		1000			66

Data obtained in cell lines transfected with human or bovine([a]) endothelin receptors. For BQ788 and 97-139 only radioligand binding data obtained in membranes are available (ET_A: SK-N-MC human neuroblastoma cell line (BQ788), and A7r5 rat aortic smooth muscle cells (97-139); ET_B: human Girardi heart (hGH) cells). For Ref. 59, pIC_{50} values were calculated to approximate IC_{50} values.

competition. Also the formation of ligand-receptor complexes with a different dissociative behaviour, depending on the ligand used, may result in complicated receptor kinetics [16, 17]. Lastly, endothelin receptors may downregulate [18] or desensitize rapidly, resulting in a possibly differentially altered response to various endothelin peptides [19].

10.3 Responses mediated by endothelin receptors

Typical ET_A receptors

ET_A receptors have often been found to mediate contractile responses in isolated smooth muscle preparations. The involvement of ET_A receptors was typically established on the basis of the relative order of potency of ET-1 and ET-3 as agonists, and on the inability of ET_B receptor selective compounds (e.g. sarafotoxin S6c or [$Ala^{1,3,11,15}$]ET-1) to act as agonists. Moreover, both BQ123 and FR139317 were generally used as ET_A receptor antagonists. Typical examples of preparations with ET_A receptors that mediate contractions include the rat [20] and guinea-pig [21] aorta (Table 2).

Typical ET_B receptors

ET_B receptors were originally considered as 'vasodilator receptors' in contrast to the vasoconstrictor ET_A receptor. Warner and colleagues [22] showed that ET-3 and ET-1 were equipotent as vasodilators, whereas ET-1 had been shown to be twenty-fold more potent as a vasoconstrictor [23]. Thus, the involvement of ET_B receptors was first established on the basis of equipotency of ET-1 and ET-3. Later, it was shown that ET_B receptors were also involved in smooth muscle contraction in blood vessels, such as the rabbit saphenous vein [24], and in the guinea-pig bronchus [21]. In these tissues the ET_A receptor antagonist BQ123 failed to block the contractile responses. The latter study also employed sarafotoxin S6c, a selective agonist for ET_B receptors. Others used [$Ala^{1,3,11,15}$]ET-1, and sometimes IRL1620 or BQ3020 as agonists. Until recently, only IRL1038 was available as a selective ET_B receptor antagonist [25, 26]. Unfortunately, the affinity for ET_B receptors was reported to be highly variable between batches, and data obtained with this compound should be considered with caution [27]. However, the recent development of the potent and selective ET_B receptor antagonist, BQ788, provided a novel tool to study involvement of ET_B receptors [28] (Table 3).

Mixed ET_A and ET_B receptor populations

In the guinea-pig trachea, BQ123 was a weak antagonist of ET-1-induced contraction; sarafotoxin S6c was a partial agonist. The contractile effect of the latter was resistant to antagonism by BQ123. Thus, it was concluded that both ET_A and ET_B receptors mediated contractile responses in the guinea-pig trachea [21]. Vasoconstriction in the isolated perfused rat kidney was also mediated by both ET_A and ET_B receptors. In this preparation, the ET_A receptor antagonists BQ123 and FR139317 caused only partial attenuation of ET-1-induced contractions, whereas the non-selective ET_A/ET_B receptor antagonist PD145065 completely

Table 2. Examples of studies in which the response (contraction) was concluded to be mediated via an ET_A receptor.

		Characterization criteria		
Species	Tissue	Agonist	Antagonist against ET-1	Ref.
Rat	Thoracic aorta	ET-1 > ET-3;	pA_2 BQ123: 6.93	20
		$[Ala^{1,3,11,15}]$ET-1: no effect		20
Rabbit	Carotid artery	ET-1 > ET-3;	pA_2 BQ123: 6.8	30
		$[Ala^{1,3,11,15}]$ET-1 and sarafotoxin S6c: no effect		30
		ET-1 > ET-3;	pK_B BQ123: 7.5	24
		sarafotoxin S6c: no effect		24
Guinea-pig	Pulmonary artery	ET-1 > ET-3	pA_2 FR139317: 6.65	67
		Sarafotoxin S6c: no effect	pK_B BQ123: 6.7	21
	Aorta	Sarafotoxin S6c: no effect	pK_B BQ123: 7.1	21
		ET-1 > ET-3;	pA_2 BQ123: 7.4	49
		$[Ala^{1,3,11,15}]$ET-1 and sarafotoxin S6c: no effect		49
	Iliac artery	ET-1 > ET-3;	pK_B BQ123: 6.6-7.2;	68
		sarafotoxin S6c: no effect	pA_2 FR139317: 5.82	68
Goat	Cerebral artery	ET-1 > ET-3	pK_B BQ123: 7.43	34
Human	Coronary artery	ET-1 > ET-3	pA_2 BQ123: 6.4-7.47	52
	Omental artery	ET-1 > ET-3	pA_2 BQ123: 7.09	26
	Pulmonary artery	Sarafotoxin S6c: no effect	pK_B BQ123: 6.2-6.8	21

'>' refers to 'is more potent than'.

Table 3. Examples of studies in which the response was concluded to be mediated via an ET_B receptor.

		Characterization criteria		
Species	Tissue, response	Agonist	Antagonist*	Ref.
Rat	Aorta relaxation	IRL1620	BQ123: no effect (IRL1620)	69
Rabbit	Pulmonary artery contraction	ET-1 = ET-3;	BQ123: no effect	30
		[Ala^{1,3,11,15}]ET-1 and sarafotoxin S6c	(ET-1, ET-3, [Ala^{1,3,11,15}]ET-1)	30
		ET-1 = ET-3; BQ3020	BQ123: no effect (BQ3020)	70
		ET-1 = sarafotoxin S6c	BQ123 and PD124893: no effect (ET-1)	43
			pA_2 BQ788: 8.4 (BQ3020)	29
	Jugular vein contraction	ET-1 = ET-3;	BQ123: no effect	30
		[Ala^{1,3,11,15}]ET-1 and sarafotoxin S6c	(ET-1, ET-3, [Ala^{1,3,11,15}]ET-1)	30
	Saphenous vein contraction	ET-1 = ET-3; sarafotoxin S6c	BQ123: no effect (Sarafotoxin S6c, ET-1)	24
Guinea-pig	Bronchus contraction	Sarafotoxin S6c	BQ123: no effect (Sarafotoxin S6c, ET-1)	21
	Trachea contraction	IRL1620		71
		ET-1 = ET-3	FR139317: no effect (ET-1, ET-2, ET-3)	67
Pig	Pulmonary artery relaxation	[Ala^{1,3,11,15}]ET-1		72
		BQ3020		70
Canine	Coronary artery constriction	Sarafotoxin S6c	BQ123: no effect (Sarafotoxin S6c)	44
Human	Bronchus contraction	Sarafotoxin S6c	BQ123: no effect (Sarafotoxin S6c, ET-1)	21

'=' refers to 'is equipotent as'; '>' refers to 'is more potent than'. *, The agonist against which the antagonist was studied is indicated between brackets.

Table 4. Examples of studies in which the response was concluded to be mediated via a mixed receptor population consisting of ET_A and ET_B receptors

		Characterization criteria		
Species	Tissue, response	Agonist	Antagonist*	Ref.
Rat	Kidney perfusion	ET-1=ET-3= sarafotoxin S6b= sarafotoxin S6c	BQ123: little effect (ET-1/sarafotoxin S6b)	73
		Sarafotoxin S6c	BQ123 and FR139317: partial attenuation (ET-1)	29
			PD145065: complete attenuation (ET-1)	29
Rabbit	Pulmonary artery	Sarafotoxin S6c > ET-1	BQ123: antagonist (high concentrations ET-1)	32
		Sarafotoxin S6c: no effect**	pA_2 BQ123: 6.6 (ET-1)**	32
	Saphenous vein contraction	Sarafotoxin S6c > ET-1 = ET-3	10 μM BQ123: antagonist (high concentrations ET-1)	32
Guinea-pig	Trachea contraction	Sarafotoxin S6c	BQ123: no effect (sarafotoxin S6c);	21
			pK_B BQ123: 6.2 (ET-1)	21
Human	Internal mammary artery contraction	Sarafotoxin S6c: partial agonist	BQ123 and FR139317: antagonists (ET-1)	51

'=' refers to 'is equipotent as'; '>' refers to 'is more potent than'. *, The agonist against which the antagonist was studied is indicated between brackets. **, After 30 min pretreatment with sarafotoxin S6c.

abolished the responses to ET-1 [29].

Even in blood vessels which were previously considered to contract via ET_B receptors exclusively, such as the rabbit saphenous vein [24] and pulmonary artery [30], a coexisting ET_A receptor mediating vasoconstriction could be demonstrated by showing that BQ123 attenuated part of the concentration response curve to ET-1 in both vessels, despite an

observed equipotency of ET-1 and ET-3 [31, 32]. The presence of ET_A receptors in the rabbit pulmonary artery was confirmed by radioligand membrane binding studies [32] (Table 4).

Atypical endothelin responses

A number of preparations yielded an agonist order of potency of ET-1 > ET-3, which would imply the involvement of ET_A receptors. However, when the effect of the ET_A receptor antagonist BQ123 was studied against both ET-1 and ET-3, it was found that BQ123 antagonized contractions to ET-3 substantially more potently than ET-1-induced contractions, suggesting the presence of different receptors [20] (Table 5A). Pierre and Clarke [33] suggested that, in the rat isolated renal artery, the BQ123-sensitive contractions to ET-3 were mediated via ET_A receptors, whereas the relatively BQ123-insensitive ET-1-induced contractile responses were mediated via non-ET_A receptors. Although this is a plausible explanation from the antagonist point of view, it is yet unclear why ET-3 recognizes an ET_A receptor not recognized by ET-1, which clearly has a higher affinity for ET_A receptors than ET-3 (Table 1). Similar observations were made when comparing the antagonist potency of BQ123 against ET-1 and sarafotoxin S6b: contractile responses of the goat cerebral artery induced by sarafotoxin S6b were antagonized more potently by BQ123 than those induced by ET-1 [34] (Table 5A). It has been argued that the reversibility of receptor binding of ET-1 is different from that of ET-3 or sarafotoxin S6b, and that this could account for the differences in antagonist potencies against these agonists [35]. However, the latter interpretation does not explain the biphasic antagonism of sarafotoxin S6b-induced contractions of the human saphenous vein by BQ123 [36], or the biphasic displacement by BQ123 of binding with 30 pM [^{125}I]-sarafotoxin S6b in the media of human coronary arteries [37]. Furthermore, it should be noted that in other investigations the antagonist potency did not differ between these particular agonists [38-40], or was even higher against ET-1 than against the other agonist [41, 42]. Thus, although the possibility of interference by complex endothelin receptor kinetics [15-19] should not entirely be disregarded, it appears that ET-1 on one hand, and sarafotoxin S6b and ET-3 on the other hand, may exert their effects via different receptors which do not fit the current classification of ET_A and ET_B receptors.

Warner and colleagues [43] observed an ET_B receptor mediating constriction of the rabbit pulmonary artery and rat stomach strip, relatively insensitive to the non-selective endothelin receptor antagonist PD142893. In contrast, PD142893 potently antagonized the ET_B receptor-mediated vasodilator effect in the perfused mesentery, which indicated

Table 5A. Examples of studies in which the response was concluded to be mediated by a single or mixed receptor population consisting of receptors characterized as partly atypical or as subtypes of ET_A receptors, by using ET_A receptor-selective compounds.

		Characterization criteria	
Species	Tissue, response	Observation	Ref.
Rat	Aorta contraction	BQ123 more potent vs. ET-3 than vs. ET-1; [Ala^{1,3,11,15}]ET-1: no effect	20
	Vas deferens increased twitch	BQ123 and PD142893 more potent vs. ET-3 and sarafotoxin S6b than vs. ET-1; sarafotoxin S6c: no effect	56,74
Goat	Cerebral artery contraction	BQ123 more potent vs. sarafotoxin S6b than vs. ET-1	34
Human	Small omental vein contraction	BQ123 more potent vs. ET-3 than vs. ET-1	26
		BQ123 more potent vs. high than vs low concentrations of ET-3; IRL1038 no effect against ET-1; sarafotoxin S6c: no effect	26 26 26
	Coronary artery contraction	BQ123 more potent vs. ET-3 than vs. ET-1	52
		BQ123 and FR139317 more potent vs. sarafotoxin S6b than vs. ET-1; [Ala^{1,3,11,15}]ET-1: no effect	53 53
	Saphenous vein contraction	BQ123 more potent vs. sarafotoxin S6b than vs. ET-1	36
		BQ123 more potent vs. high than vs. low concentrations of sarafotoxin S6b	36
	Umbilical artery	BQ123 more potent vs. sarafotoxin S6b than vs. ET-1	54

receptor heterogeneity among ET_B receptors [43]. Also a study in swine pulmonary artery revealed differences between ET_B receptors mediating contraction and ET_B receptors mediating endothelium-dependent relaxation. Only the latter receptor was sensitive to antagonism by the ET_B receptor antagonist IRL1038 [25]. As was mentioned above, it should however be noted that questions have arisen over the use of IRL1038 as an ET_B receptor antagonist [27], and these experiments need verification using alternative antagonists with affinity for ET_B receptors. Radioligand binding studies in canine coronary artery membranes also indicated the possibility of ET_B receptor subtypes [44]. These binding sites

Table 5B. Examples of studies in which the response was concluded to be mediated by a single or mixed receptor population consisting of receptors characterized as partly atypical or as subtypes of ET_B receptors, by using ET_B receptor-selective compounds.

		Characterization criteria	
Species	Tissue, response	Observation	Ref.
Rat	Stomach strip contraction	Contraction to sarafotoxin S6c (more potent than ET-1) weakly antagonized by PD142893	43
	Perfused mesentery	Dilatation to sarafotoxin S6c (equipotent ET-1) strongly antagonized by PD142893	43
	Atrium contraction	ET-1, ET-3, sarafotoxin S6b equipotent; sarafotoxin S6c and [$Ala^{1,3,11,15}$]ET-1 no effect	45
Pig	Pulmonary vein contraction*	Isopeptide non-selective receptor, resistant to IRL1038	25
	Pulmonary artery relaxation*	Isopeptide non-selective receptor, sensitive to IRL1038	
	Coronary artery contraction	Sarafotoxin S6c sensitive receptor recognizes ET-3, but not ET-1 or sarafotoxin S6b	48
		Sarafotoxin S6c and [$Ala^{1,3,11,15}$]ET-1 sensitive receptor [pK_B BQ123: ≈5 (ET-1)], and another receptor resistant to BQ123	49

*, The receptor affinity and selectivity for ET_B receptors of IRL1038 have been described to be highly variable. Therefore, these data must be interpreted with caution[27].

had either high (ET_BH) or low (ET_BL) affinity for both ET-1 and ET-3. In addition, the ET_BH site showed high affinity for sarafotoxin S6c, but not for BQ123, whereas the ET_BL site had moderate affinity for both sarafotoxin S6c and BQ123. Coronary vasoconstriction induced by sarafotoxin S6c was insensitive to BQ123, indicating involvement of the putative ET_BH receptor. No functional correlate for the ET_BL site is known at present [44]. In the rat left atrium, equipotent contractile responses to ET-1, ET-2, ET-3 and sarafotoxin S6b were observed, indicating the involvement of ET_B receptors. However, the ineffectiveness of the ET_B receptor agonists [$Ala^{1,3,11,15}$]ET-1 and sarafotoxin S6c would suggest the involvement of receptors other than conventional ET_B receptors [45]. Similarly,

xenopus laevis liver membranes revealed a binding site with identical affinity for ET-1 and ET-3. As expected for ET_B receptors, BQ123 was ineffective displacing [^{125}I]-ET-1 labelling of this site, but sarafotoxin S6c was ineffective as well, suggesting the presence of a subtype of ET_B receptors [46].

The nature of endothelin receptors mediating contraction of the porcine coronary artery is still controversial, but part of the receptor population does not appear to correspond to either ET_A or ET_B receptors. Ihara and co-workers [47] observed ET-1-induced contractile responses sensitive to antagonism by BQ123 (pA_2 7.4), which were thus considered to be mediated via ET_A receptors. The small BQ123 non-sensitive part of the concentration response curve to ET-1 was assigned to ET_B receptors. Further studies agreed on the ET_A receptor component based on the agonist order of potency, but also observed a receptor which recognized sarafotoxin S6c and ET-3, but not ET-1 and sarafotoxin S6b [48]. Later, it was shown that the contractile effects of both sarafotoxin S6c and [Ala^{1,3,11,15}]ET-1 were likely to be mediated via the same ET_B receptor, whereas a non-ET_A, non-ET_B type of receptor contributed to the contractile response induced by ET-1 [49] (Table 5B).

Endothelin receptors in human blood vessels

Endothelin receptors mediating contractions of human isolated blood vessels were recently reviewed by Davenport and Maguire [50]. Although the contractile responses may be mediated via typical ET_A receptors (perhaps in addition to ET_B receptors [51]), there are several reports focusing on non-ET_A, non-ET_B receptors in human blood vessels.

In parallel with the rat aorta [20] and the goat cerebral artery [34] (Table 5), BQ123 has been observed to be more potent against ET-3- and sarafotoxin S6b-induced contractions than against ET-1-induced contractile responses in the human isolated saphenous vein [36], coronary artery [52, 53], umbilical artery [54] and in small omental veins [26]. Recent data obtained in the human isolated coronary artery suggest that the same discrepancy between ET-1- and sarafotoxin S6b-induced contractile responses is also observed with other ET_A receptor antagonists such as FR139317 [53].

It is yet unclear whether the ET_B receptor plays a significant role in vasoconstriction of human blood vessels. Indeed the endogenous ligand ET-3 is a less potent vasoconstrictor agonist than ET-1. Moreover, both ET_B receptor agonists BQ3020 and [Ala^{1,3,11,15}]ET-1 hardly contracted the human isolated coronary artery [50]. Sarafotoxin S6c, however, induced contractile responses in some (but not all) coronary artery [50], internal mammary artery [51] or saphenous vein [55] segments. Although this may be due to a relatively low ET_B receptor density [50], these observations could also be related to the isopeptide non-

selective ET_B receptors, with low affinity for the ET_B receptor agonists [$Ala^{1,3,11,15}$]ET-1 or sarafotoxin S6c [45, 46].

10.4 Concluding remarks

The above mentioned studies show us that the current ET_A and ET_B endothelin receptor classification will have to be extended. A number of responses fit the present criteria for the ET_A receptor, but ET_A receptor antagonists (like BQ123 and FR139317) can sometimes be shown to be more potent against ET-3 [20, 26, 52] or sarafotoxin S6b [34, 36, 53, 54] than against ET-1, indicating further heterogeneity of endothelin receptors. One may classify these receptors as subtypes of the ET_A receptor, since ET_A receptor antagonists are moderately or highly potent in these assays, whereas ET_B receptor agonists are usually inactive (Table 5A). However, since it has only been possible to detect this heterogeneity in assays in which both receptors mediate the same effect, a detailed operational analysis of the *individual* receptors has not yet been established, and a conclusive classification of these receptors is therefore best postponed.

ET_B receptors also appear to be heterogeneous since the relaxant but not the contractile responses were antagonized by PD142893 [43]. It should be noted that only the affinity for the antagonist is to be used as a criterium for pharmacological receptor classification, and not whether the receptor mediates contraction or relaxation [6, 7]. In view of the effect of ET_B receptor agonists and the ineffectiveness of BQ123, it would appear that these receptors may be designated ET_{B1} (PD142893 sensitive) and ET_{B2} (PD142893 insensitive) receptor subtypes. Whether additional ET_B receptor subtypes exist, e.g. in canine coronary artery [44], or whether other atypical observations are related to the proposed ET_{B1} or ET_{B2} receptor subtypes, remains to be resolved (Table 5B).

The above mentioned novel experimental data are not yet sufficient to provide a conclusively extended endothelin receptor nomenclature. Indeed additional data on transductional mechanisms and possibly on the sequence of the corresponding DNA are eagerly awaited, and the use of more agonists and antagonists in functional studies is vital [6, 7]. For now we would however point out that in particular the current basic criterium of the potency difference of ET-1 and ET-3 [8] should be applied with the utmost restraint. In some smooth muscle preparations, ET-1 is clearly more potent than ET-3, suggesting the involvement of ET_A receptors. However, considering the observed antagonist potency

differences [20, 26, 52], the two peptides may in fact induce contractions via different receptors (Table 5A). In other tissues ET-1 and ET-3 are equipotent which would traditionally point at ET_B receptors. However, ET_B receptor agonists may in some cases be inactive [45], and detailed analysis using ET_A receptor antagonists has been used to demonstrate the presence of an additional ET_A receptor [31, 32]. Given the relatively incomplete understanding of endothelin receptors and the limited number of selective receptor ligands, the use of a wide spectrum of both agonists and antagonists is required for endothelin receptor characterization. The non-peptide receptor antagonists, that are now becoming available, will certainly help the process towards a conclusive, more detailed endothelin receptor classification.

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Appendix Chapter 10; Chemical names

BQ123: cyclo(D-Trp-D-Asp-Pro-D-Val-Leu-)

BQ3020: $Ala^{11,15}$-Ac-ET-1[6-21]

FR139317: 2(R)-[2(R)-[2(S)-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indolyl)]propionyl]amino-3-(2-pyridyl)proprionic acid

IRL1620: Suc-[Glu^{9},$Ala^{11,15}$]ET-1[8-21]

IRL1038: Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp

PD145065: Ac-(5H-dibenzyl[a,d]cycloheptane-10,11-dihydro-glycine)-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp

PD142893: Ac-(3,3-D-diphenylalanine-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp

BQ788: N-cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-norleucin

Ro-46-2005: 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(3-methoxy-phenoxy)-4-pyrimidinyl]-benzenesulphonamide

Bosentan: 4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide

SB 209670: (+)-(1S, 2R, 3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)-indane-2-carboxylic acid

BMS-182874: 5-(dimethylamino)-N-(3,4,-dimethyl-5-isoxazolyl)-1-naphtalenesulfonamide

97-139: 27-O-3-[2-(3-carboxy-acryloyl-amino)-5-hydroxyphenyl]acryloyloxy-myricerone sodium salt

Chapter 11

Arginine vasopressin-induced responses of the human isolated coronary artery: effects of non-peptide receptor antagonists*

Summary - Contractions to arginine vasopressin and the effect of non-peptide vasopressin receptor antagonists were studied in the human isolated coronary artery. The contractile response to AVP was antagonized by both the putative V_1 receptor antagonist, SR 49059, and by the reported V_2 receptor antagonist, OPC-31260 (pA_2: 9.76 and 7.31, respectively). In contrast to its reported high affinity for rat V_1 receptors, OPC-21268 antagonized the response to AVP only in a concentration of 3 μM (apparent pK_B: 5.6). The antagonist potency order (SR 49059 > OPC-31260 > OPC-21268) corresponds to the reported affinity order for the cloned human V_1 receptor. Therefore, the affinity of OPC-21268 appears confined to rat, but not human V_1 receptors, which raises the question whether these receptors are species homologues or separate receptor entities.

11.1 Introduction

Vasopressin and oxytocin are related hormones, which are released from the pituitary. Although vasopressin and oxytocin only differ in the third and eighth amino acid position, their relative potencies are remarkably different in functional assays. Vasopressin primarily contributes to cardiovascular homoeostasis by inducing vasoconstriction and by stimulating renal water absorption, whereas oxytocin acts as an agonist which stimulates milk-ejection and parturition (review, see Ref. 1). Pigs have a lysine in the eighth position of vasopres-

*, *Based on:* Bax, W.A., Van der Graaf, P.H., Bos, E., Nisato, D. and Saxena, P.R. (1995) Arginine vasopressin-induced responses of the human isolated coronary artery: Effects of non-peptide receptor antagonists (submitted).

sin instead of arginine in most other mammals [2]. For that reason human vasopressin is often specified as arginine vasopressin (Figure 1).

The effects of AVP are mediated via vasopressin V_1 and V_2 receptors, respectively. In general, V_1 receptors stimulate phospholipase C, resulting in the production of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG), whereas V_2 receptors activate adenylyl cyclase. The human V_1 and V_2 receptor have both been cloned. The nucleotide coding sequence identity of the human V_1 receptor with the rat liver V_1 receptor, the human oxytocin receptor, the human V_2 receptor, and the rat V_2 receptor is 83%, 68%, 62%, and 65% respectively [3, 4, 5]. The existence of an additional vasopressin receptor subtype was postulated in the rat adenohypophysis. This receptor has been designated V_3, or V_{1b} as opposed to the V_1 or V_{1a} receptor on liver, smooth muscle, and platelets [6]. The recent elucidation of the aminoacid structure of a vasopressin receptor from the pituitary appeared to indicate that this receptor did not diverge from the human V_{1a} receptor, but evolved from a common ancestor at an earlier stage during the evolution. At the nucleotide level, the strongest homology was found between this receptor and the human oxytocin receptor. Therefore, the designation as V_3 receptor appears more appropriate than V_{1b} [6a].

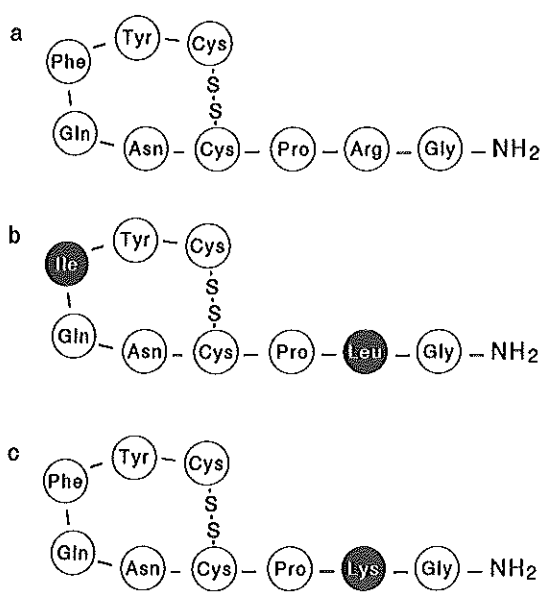

Figure 1. Structure of arginine vasopressin (a), oxytocin (b), and porcine lysine vasopressin (c). Amino acids different from the amino acid sequence of arginine vasopressin are highlighted.

Antagonists for vasopressin receptors may be beneficial in the treatment of ischemic heart disease, hypertension, and congestive heart failure [6b, 6c]. Recently, two non-peptide vasopressin antagonists were developed with apparent selectivity for V_1 receptors: OPC-21268 [7], and SR 49059 [8, 9]. Another non-peptide receptor antagonist, OPC-31260, was reported selective for V_2 receptors [10]. OPC-21268 produced hypotension in spontaneously hypertensive rats [11], and attenuated AVP-induced vasoconstrictor responses in the human forearm *in vivo* [12]. OPC-31260 had a diuretic effect in men, which was almost equipotent when compared to the effect of furosemide [13].

Liu and colleagues [14] showed that OPC-21268 did not antagonize AVP-induced contractions of the human isolated internal mammary artery, whereas both the putative V_1 receptor antagonist, [d(CH_2)$_5$sarcosine^7]AVP (SAVP), and the putative V_2 receptor antagonist, OPC-31260, acted as competitive antagonists of this response. In the present study, we compared the effect of SR 49059, OPC-21268, and OPC-31260, as antagonists against AVP-induced contractions of the human isolated coronary artery.

11.2 Materials and methods

Tissue preparation and experimental protocol

Hearts were obtained from 14 organ donors, who had died of non-cardiac disorders (5 cerebrovascular accident, 7 polytrauma, 2 cerebral hypoxia; 9 male, 5 female; age 10-54 years). The hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services Foundation) after removal of the valves for transplantation purposes. The right epicardial coronary artery was placed in a Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2 (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_2$ 25, and glucose 8.3; pH: 7.4). 4 mm rings segment were suspended in 15 ml organ baths containing the Krebs bicarbonate solution (37 °C). The endothelium was left intact as verified by observing relaxation to substance P (1 nM) after precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). In some ring segments the endothelium was removed using a cotton swab. Changes in tension were recorded using a Harvard isometric transducer. Tissues were stretched to a tension of 20 mN, and were subsequently exposed to 100 mM K^+. After a washout period of 30 minutes, segments were incubated with vehicle (controls) or an antagonist for thirty minutes, before a cumulative concentration-response curve was obtained.

Analysis of Data

Curves were analyzed by means of a computerized curve fitting technique [15] to obtain the pD_2 value (-log [EC_{50}], mol/l). pA_2 values were calculated according to Arunlakshana and Schild [16]. All data are presented as mean ± s.e.m.. pD_2 and maximal effect (E_{MAX}) of agonist-induced contractions in the absence and presence of an antagonist were compared using a paired Student's t-test. A *P* value less than 0.05 was assumed to denote a significant difference.

Compounds

Prostaglandin $F_{2\alpha}$ (tris salt), substance P acetate, and [Arg^8]-vasopressin (acetate salt) (purchased from Sigma Chemical Co., St. Louis, USA), were dissolved in distilled water. OPC-21268 (1-{1-[4-(3-acetylaminopropoxy)benzoyl]-4-piperidyl}-3,4-dihydro-2(1*H*)-quinolinone), OPC-31260 ([5-dimethylamino-1-{4-(2-methylbenzoylamino)benzoyl}-2,3,4,5-tetrahydro-1*H*-benzazepine]), and SR 49059 ((2S) 1-[(2R 3S)-5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenze ne-sulfonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl]-pyrrolidine-2-carboxamide) were synthesized by Sanofi Recherche, Montpellier, France, and dissolved in dimethylsulphoxide (DMSO) at a concentration of 1 mM, and subsequently diluted in distilled water.

11.3 Results

100 mM K^+ caused a mean contractile response of endothelium-intact coronary artery segments of 40 ± 4 mN (n=14). In these preparations, 1 nM substance P caused 73 ± 7% relaxation after precontraction with 1 µM $PGF_{2\alpha}$. Removal of the endothelium reduced relaxation to 10 ± 7% of the response to $PGF_{2\alpha}$ (n=8).

AVP induced concentration-dependent contractions (pD_2: 9.25 ± 0.15; E_{MAX}: 11.8 ± 1.8% of the response to 100 mM K^+; n=14). Removal of the endothelium did not result in a significantly altered response to AVP (pD_2: 9.30 ± 0.32; E_{MAX}: 14.8 ± 4.3%; n=8). Furthermore, we did not observe relaxation after adding 0.1-1 nM AVP to coronary artery segments (n=3) precontracted with 0.1 µM $PGF_{2\alpha}$ (precontraction: 15.4 ± 1.1% of the response to 100 mM K^+). By contrast, addition of 1 nM AVP induced an additional contractile response of 6.7 ± 2.9% of the response to 100 nM K^+. All segments subsequently relaxed completely to 1 nM substance P (Figure 2).

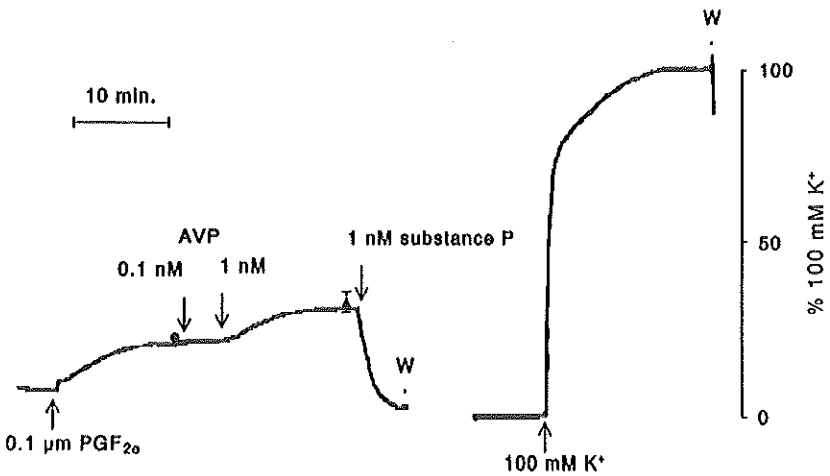

Figure 2. Original tracing of the response of an endothelium-intact coronary artery segment. No relaxation was observed after adding 0.1-1 nM AVP to the coronary artery segment precontracted with 0.1 µM $PGF_{2\alpha}$. The segment subsequently relaxed completely to 1 nM substance P. The mean response (± s.e.m.) of 3 segments is also indicated (●, ▲; expressed as percentage of the reference contractile response to potassium, 100 mM).

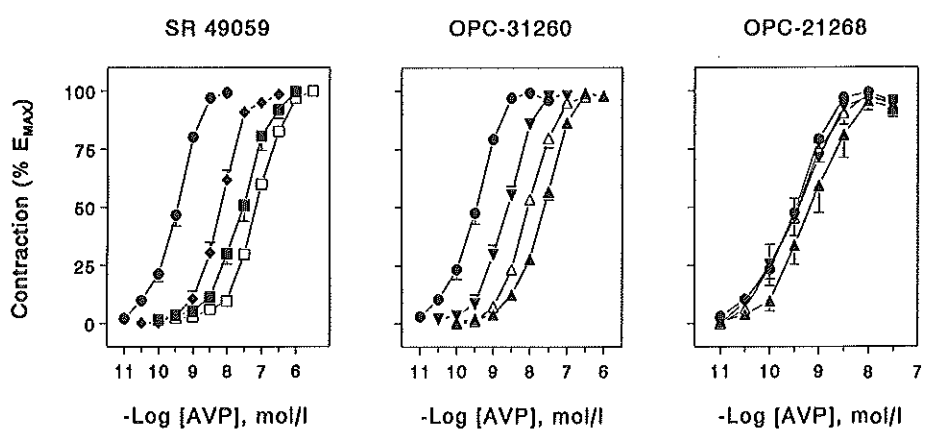

Figure 3. Contractions of the human isolated coronary artery to AVP in the absence (control: ●) or presence of the vasopressin receptor antagonists SR 49059, OPC-31260 and OPC-21268 (n=5-7). Concentrations of receptor antagonists were 3 nM (♦), 0.01 µM (■), 0.03 µM (□), 0.1 µM (▽), 0.3 µM (▼), 1 µM (△) or 3 µM (▲).

In endothelium-intact coronary artery segments, SR 49059 (3-30 nM) and OPC-31260 (0.3-3 μM) induced a concentration-dependent parallel rightward shift of the AVP concentration response curve, with associated pA_2 values of 9.76 ± 0.16 and 7.31 ± 0.18, respectively (n=7, Figure 3). Schild analysis revealed that the slopes for SR 49059 and OPC-31260 were 1.08 ± 0.10 and 1.07 ± 0.07, respectively (not significantly different from unity), indicating that these compounds antagonized the response to AVP in a competitive manner. OPC-21268 caused a significant rightward shift only in the highest concentration used (3 μM). An apparent pK_B of 5.6 ± 0.3 (n=6) was calculated, assuming a Schild-plot slope of unity.

11.4 Discussion

The most significant observation of the present study is the discrepancy between the two putative V_1 receptor antagonists SR 49059 and OPC-21268; SR 49059 was a potent receptor antagonist (pA_2: 9.76), whereas OPC-21268 had no effect in concentrations up to 1 μM. Even the putative V_2 receptor antagonist OPC-31260 was more potent than OPC-21268. The data regarding OPC-21268 appear difficult to interpret when considering a pK_I value of 7 in rat liver membranes (V_1 receptor model) [7]. However, the present data are in agreement with the reported affinity for the recently cloned human V_1 receptor (Table 1). Therefore, affinity for rat V_1 receptors is not predicative of functional antagonist potency at human V_1 receptors. The effect of OPC-31260 may be explained by its additional affinity for the human V_1 receptor (Table 1). The observed pA_2 value of 7.31 would rule out involvement of the putative V_3 receptor, for which OPC-31260 appeared to have supra-micromolar affinity [6a].

OPC-21268 was previously shown to attenuate AVP-induced vasoconstriction of the human forearm [12]. This effect may be explained by (*i*) a high plasma concentration of the antagonist (>1 μM), (*ii*) conversion into an unknown, more active metabolite, or (*iii*) by an effect at other receptors than the receptor in the human coronary artery.

Table 1. Comparison of the affinity of vasopressin antagonists in receptor binding assays of rat and human V_1 receptors, compared with the potency as receptor antagonist in the human coronary artery.

	pK_i value (-log [K_i (mol/l)]) binding assay		pA$_2$ value in functional assay
Compound	Rat V_1 receptor	Human V_1 receptor	Human coronary artery
SR 49059	9.10[1]	8.49[1], 8.89[2]	9.76
OPC-21268	7.21[1], 7.24[3]	4.57[1], 4.25[3]	5.6
OPC-31260	6.54[1], 6.67[3]	6.60[1], 6.47[3]	7.31

[1], Data from Ref. 9 (liver membranes); [2], Data from Ref. 3 (cloned receptors expressed in cell lines); [3], Data from Ref. 4 (cloned receptors expressed in cell lines).

It was observed that the maximal effect of AVP reached only 12% of the contractile response to 100 mM K^+. This appears lower than in somewhat smaller blood vessels such as the human internal mammary artery (approx. 50% of the maximum response to noradrenaline) [14], or the human cerebral artery (approx. 100% of the response to 100 mM K^+) [17]. On the other hand, we are not aware of endogenous hormones that contract the human isolated coronary artery with similar or higher potency than AVP.

It was also investigated whether the response to AVP was modulated by the presence of endothelium. Although removal of the endothelium resulted in a slightly, but non-significantly enhanced contractile response to AVP, we observed no endothelium-dependent relaxation to AVP in endothelium-intact vessel segments precontracted with $PGF_{2\alpha}$ (Figure 2). The present data therefore provide no evidence for the involvement of endothelial vasopressin receptors mediating relaxation, as was also concluded in recent studies in the human cerebral and omental artery [17, 18].

In summary, AVP induces contraction of the human isolated coronary artery via an OPC-21268-insensitive receptor. The functional characteristics of this receptor are congruent with the binding characteristics of the recently cloned human V_1 receptor, which, in contrast to the rat cloned V_1 receptor, has low affinity for OPC-21268. Thus, it may be concluded that rat and human V_1 receptors have a distinct pharmacological profile, which raises the question whether these receptors are species homologues or separate receptor entities.

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Ancient Egyptian illustration. The heart is balanced as a witness of life.

Part 4

Synopsis

Chapter 12

Summary, general discussion, and implications for future research

12.1 Summary of the thesis

Chapter 1 provided an overview of clinical aspects of ischemic heart disease. Ischemic heart diseases may clinically be subdivided in stable angina, unstable angina, acute myocardial infarction, and variant angina. These disorders are part of a spectrum of related disorders, in which the supply of oxygen does not meet the demand. This usually results in chest pain (angina), and a number of additional physical, chemical, and electrophysiological symptoms. The pathophysiology involves *passive* luminal obstruction of the blood vessel, via thrombus plugging or plaque formation, and *active* vasoconstriction induced by disbalance of vasomotor tone. The contribution of active vasoconstrictor obstruction may vary from very little in case of fixed obstruction in stable angina, up to almost hundred percent in case of variant angina pectoris. Angina can sometimes be caused by treatment with vasoconstrictor drugs, like the anti-migraine drugs, ergotamine and sumatriptan. Treatment of ischemic heart disease is aimed at reducing myocardial ischemia and related anginal pain, and at early reperfusion and prevention of haemodynamic complications in myocardial infarction. Large scale clinical trials are being undertaken to establish the best choice of treatment for different groups of patients.

Chapter 2 described the physiology and pathophysiology of vasomotor tone. Disturbed vasomotor tone is believed to play a significant role in the above mentioned clinical syndromes of ischemic heart disease. Vasomotor tone is the outcome of contraction and relaxation, and is affected by processes within the vascular smooth muscle and the endothelium. Smooth muscle contraction and relaxation result primarily from the interaction of actin and myosin protein chains. These are regulated via a large number of receptors, enzymes, ion-pumps, and ion-exchangers, many of which influence the concentration of cytosolic calcium, or the sensitivity of the contractile apparatus. The endothelium is now known to be an important determinant of vasomotor tone, via the release of relaxant (endothelium-derived relaxing factor, EDRF) or contractile stimuli

(endothelium-derived contracting factor, EDCF), which can both have effects on the underlying smooth muscle. A disturbance of the equilibrium between relaxation and contraction can lead to decreased flow, and subsequent decreased supply of oxygen to the myocardium. Disturbance of equilibrium is particularly likely in atherosclerotic blood vessels, in which the dilatory component, mediated by the endothelium, is decreased. Also endothelial injury after balloon angioplasty, and serum lipoproteins and cholesterol may be involved in disruption of the contraction-dilatation equilibrium.

Chapter 3 provided an overview of 5-hydroxytryptamine, its receptor subtypes and their functional and therapeutical relevance in the cardiovascular system. The development and rationale of the early 'M' and 'D' receptor classification up to the present IUPHAR classification of receptors for 5-HT was described. Furthermore, it was pointed out that, although exogenous 5-HT may have profound effects on the cardiovascular system, antagonism of endogenous 5-HT has thus far not been very successful in the treatment of cardiovascular diseases. The use of 5-HT receptor agonists and antagonists in cardiovascular diseases has remained limited to the treatment of migraine. The disappointing results obtained with 5-HT receptor antagonists in diseases that involve coronary vasospasm may point at overestimation of the role of 5-HT in the disease process, or at the use of a receptor antagonist aimed (in part) at the wrong receptor.

In **Chapter 4,** the receptors mediating the contractile effect of 5-HT on the human isolated saphenous vein were investigated, using a number of 5-HT receptor agonists and antagonists. The rank order of agonist potency was 5-CT ≈ 5-HT > methysergide ≈ sumatriptan ≈ α-methyl-5-HT ≈ RU 24969 ≈ DOI > 2-methyl-5-HT > 8-OH-DPAT. The 5-HT_2 receptor antagonist, ketanserin, caused a rightward shift of the upper part of the concentration-response curve of 5-HT and 5-CT, while causing a parallel rightward shift of the concentration-response curves of the selective 5-HT_2 receptor agonists α-methyl-5-HT and DOI. Furthermore, contractions to 5-HT were antagonized by methysergide, methiothepin, ICS 205-930 (tropisetron) and flesinoxan. The responses to the selective 5-HT_1 receptor agonist sumatriptan were antagonized by methiothepin, metergoline, rauwolscine, and cyanopindolol, but not by ketanserin. A high correlation was observed between the functional pD_2 and pK_B values of 5-HT_1-like receptor agonists, and antagonists vs. sumatriptan, compared to their reported binding affinities for the 5-HT_{1D} receptor in radioligand binding assays of human or calf brain membranes. It is therefore concluded that contractions of the human isolated saphenous vein, induced by 5-HT, are

mediated by 5-HT_2 receptors as well as by a 5-HT_1-like receptor resembling the 5-HT_{1D} subtype found in brain membranes.

In 3 patients, undergoing heart transplantation, saphenous vein, which had previously functioned as a coronary bypass graft for 6-11 years, was dissected out from the heart. Light microscopy revealed profound intimal thickening in the grafted veins, and contractions to potassium, 5-HT and sumatriptan were significantly smaller (E_{MAX}, mN). However, both E_{MAX}, calculated as percentage of potassium-induced contractions, and pD_2 values for 5-HT and sumatriptan were similar to those found in the veins obtained directly from the lower leg.

In **Chapter 5** we investigated contractile responses of the human isolated coronary artery to 5-HT, human washed platelets, sumatriptan, and ergotamine. 5-HT and platelets caused contractile responses which were attenuated by ketanserin (1 μM). In the presence of ketanserin (1 μM), the adrenoceptor antagonists with accessory 5-HT_1 receptor affinity, rauwolscine and cyanopindolol, caused concentration-dependent additional antagonism against leftover contractions induced by low (≤ 1 μM) concentrations of 5-HT. Sumatriptan-induced contractions were antagonized to a similar extent by both rauwolscine and cyanopindolol and also by metergoline, but not by ketanserin. The order of potency of antagonists against sumatriptan resembled the order reported for the human saphenous vein 5-HT_{1D}-like receptor. Against platelet-induced contractile responses we observed only a non-significant additional antagonism by cyanopindolol or rauwolscine. It was concluded that, although 5-HT_2 receptors predominantly mediate 5-HT-induced contractions, the 5-HT_1-like receptor appears to play a role in coronary vasospasm caused by low concentrations of 5-HT.

In addition, we compared the contractile effect of sumatriptan and ergotamine. Ergotamine contracted the human isolated coronary artery with approx. hundred-fold higher potency than sumatriptan. Moreover, the E_{MAX} of ergotamine was twice that of sumatriptan.

In **Chapter 6** the contractile effect of human washed platelets on the human isolated coronary artery was investigated. Platelets (10^9-3.10^{10}/l) elicited concentration-dependent contractile responses of the coronary artery segments. The contractile response tended to be decreased in vessel segments with histological signs of early atherosclerosis. Contraction was significantly attenuated after pretreatment of the vessel segments with ketanserin, or the TxA_2 receptor antagonist SQ30741. Platelets obtained from the same platelet-donors after taking aspirin (40 mg/day for 7-13 days) caused significantly lower

contractile responses, associated with an almost selective inhibition of the synthesis of thromboxane measured in the organ bath solution. The amount of 5-HT secreted in the organ bath remained unaltered, which explains why ketanserin significantly attenuated the residual contractile responses caused by platelets obtained from aspirin-treated subjects, whereas SQ30741 caused minor, non-significant additional attenuation. The results of the study therefore suggested that additional antagonism of the contractile 5-HT receptors in the coronary artery may increase the efficacy of low dose aspirin *in vivo*.

In the experiments described in **Chapter 7**, we investigated endothelin (ET) receptors in the human coronary artery, and in ventricular and atrial muscle, using quantitative receptor autoradiography. Displacement of [^{125}I]sarafotoxin S6b (30 pM)- and [^{125}I]ET-1 (30 pM)-labelled binding sites was studied using ET-1, the ET_A receptor selective ligand BQ-123, and the ET_B receptor selective ligand [Ala^{1,3,11,15}]ET-1. Specific binding was more dense in atrium and coronary artery than in ventricular muscle. In the coronary artery, binding was especially dense in the media. ET-1 displaced [^{125}I]ET-1 and [^{125}I]sarafotoxin S6b monophasically in atrium, ventricle and coronary artery. [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1 and [^{125}I]sarafotoxin S6b-labelled sites biphasically in the ventricle and in the atrium. In the human coronary artery, [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]ET-1-labelled sites monophasically (ET-1 > BQ-123 > [Ala^{1,3,11,15}]ET-1). By contrast, [Ala^{1,3,11,15}]ET-1 and BQ-123 displaced [^{125}I]sarafotoxin S6b-labelled coronary artery sites biphasically. The low-affinity site for [Ala^{1,3,11,15}]ET-1 and BQ-123 corresponded to the binding site observed using [^{125}I]ET-1. These data indicated that both [^{125}I]ET-1 and [^{125}I]sarafotoxin S6b-labelled ET_A and ET_B binding sites in human ventricular and atrial muscle. In the human coronary artery, both radioligands labelled ET_A binding sites, but [^{125}I]sarafotoxin S6b also labelled a non-ET_A, non-ET_B binding site with relatively high affinity for both BQ-123 and [Ala^{1,3,11,15}]ET-1.

In view of the data obtained in Chapter 7, we set out to obtain *functional* evidence of corresponding receptor heterogeneity. In **Chapter 8**, we therefore investigated the effect of the ET_A receptor antagonist BQ-123 on contraction of the human isolated saphenous vein induced by ET-1 or sarafotoxin S6b. Contraction to ET-1 was not affected by BQ-123 (0.1-1 μM). By contrast, BQ-123 (0.1 - 1 μM) biphasically attenuated contractions to sarafotoxin S6b. These data indicated that *(i.)* ET-1 induced contractions of the human saphenous vein via a BQ-123-insensitive receptor and that *(ii.)* contractions to

sarafotoxin S6b were mediated in part via a receptor, different from the receptor mediating contraction to ET-1.

In **Chapter 9**, we characterized endothelin receptors, that mediate contraction of the human isolated coronary artery. The order of agonist potency (pD_2) in endothelium-intact coronary artery ring segments was: ET-1 ≈ sarafotoxin S6b > big-ET-1 ≈ ET-3. [$Ala^{1,3,11,15}$]ET-1 (an ET_B receptor agonist) caused significant contraction at 1 μM only, whereas 0.3 μM big-ET-3 had no effect. Removal of the endothelium in ring segments did not affect the contractile response to ET-1 or to sarafotoxin S6b. After a full concentration response curve with ET-1 or sarafotoxin S6b, further contractions of the endotheliumintact coronary artery segments could only be achieved by applying ET-1 in segments exposed to sarafotoxin S6b, and not the reverse. 0.1 μM BQ-123 antagonized contractions of endothelium-intact ring segments induced by sarafotoxin S6b (pK_B: 7.86). Only 10 μM BQ-123 antagonized contractions induced by ET-1 (pK_B: 5.75). FR139317 was also more potent against sarafotoxin S6b than against ET-1. [$Ala^{1,3,11,15}$]ET-1 (1 μM) had no effect on the contractile response to ET-1 or to sarafotoxin S6b. Compared to ring preparations with intact endothelium, the pD_2 of ET-1 increased in strip preparations with intact endothelium, and 1 μM BQ-123 caused a rightward shift of the ET-1-induced concentration response curve in strip preparations (pK_B: 6.62). Contractile responses to big-ET-1 of endothelium-intact coronary artery segments were attenuated in the presence of 100 μM phosphoramidon, indicating conversion of big-ET-1 to ET-1 within the coronary artery segment. It was concluded that ET-1 and sarafotoxin S6b contract the human isolated coronary artery via different receptors, which can probably be best characterized as subtypes of the ET_A receptor. Furthermore, it was demonstrated that the type of preparation (ring or strip) may affect the potency of ET-1 as an agonist, and of BQ-123 as an antagonist.

In **Chapter 10**, we reviewed typical and atypical endothelin receptors, and compared experimental evidence for endothelin receptor subtypes to the present official IUPHAR classification for endothelin receptors. It was shown that the present criteria are insufficient to characterize all endothelin receptors. Emphasis was put on the need for rigorous classification criteria, simultaneously applying operational, transductional, and structural criteria. Although experimental data showed that the present classification may be insufficient, it was also made clear that there is not yet sufficient evidence to conclusively replace the present classification with a novel endothelin receptor scheme.

In **Chapter 11** we studied the effect of nonpeptide receptor antagonists to arginine vasopressin-induced contractions of the human isolated coronary artery. The contractile response to AVP was antagonized by both the putative V_1 receptor antagonist, SR 49059, and by the reported V_2 receptor antagonist, OPC-31260 (pK_B: 9.76 and 7.31, respectively). In contrast to its reported high affinity for rat V_1 receptors, OPC-21268 antagonized the response to AVP only in a concentration of 3 μM (apparent pA_2: 5.6). The antagonist potency order (SR 49059 > OPC-31260 > OPC-21268) corresponded to the reported affinity order for the cloned human V_1 receptor. Therefore, the affinity of OPC-21268 appeared confined to rat, but not human V_1 receptors, which raised the question whether these receptors are species homologues or separate receptor entities.

12.2 General discussion

The nature of 5-HT receptors mediating contraction of human blood vessels

Most human blood vessels contract to 5-HT mediated via a mixed receptor population of 5-HT_{2A}- and 5-HT_1-like receptors. The nature of the latter receptor has remained the subject of considerable debate. Characterization of a functional receptor is facilitated by elucidation of the molecular structure of the receptor. This enables identification of regions where the receptor is expressed, and is essential for a rational design of agonists and antagonists, aimed at that particular receptor. The exact nature of the vascular, contractile 5-HT_1 receptor (also called: 5-HT_{1X}, 5-HT_1-like, or 5-HT_{1D}-like) has remained unresolved, and evidence obtained even before the cloning era, suggests that this receptor is heterogeneous in nature (Chapter 3, Table 4). Recently, it was suggested that 5-$HT_{1D\beta}$ receptors may be present in the coronary artery [1, 2] and in the pial arteriole [3] of man. However, canine coronary arteries were found to contract via 5-$HT_{1D\alpha}$ receptors [4]. Conclusions on the presence of the 5-$HT_{1D\beta}$ receptor in human coronary arteries were based essentially on the single fact that ketanserin did not attenuate sumatriptan-induced contractile responses, which had been known for some years [5-7]. Unfortunately, alternative agonists or antagonists with more pronounced selectivity for any of these receptors, have not been made available. However, it should not be ruled out that 'the 5-HT_1-like receptor' consists of a mix of several 5-HT_1 receptor subtypes, which may mediate contraction as well as relaxation. Indeed, a receptor previously classified as a 5-HT_1-like receptor was shown to mediate relaxation via an endothelium-independent pathway [8]. This receptor was classified as orphan 5-HT receptor, because of its positive linkage to adenylyl cyclase [9],

in contrast to the negative linkage of 5-HT_1 receptor subtypes to adenylyl cyclase [10]. It was suggested that this receptor may be similar to the 5-HT_7 receptor subtype, which has a somewhat similar ligand affinity pattern, and which is also coupled positively to adenylyl cyclase [10, 10a, 10b]. mRNA for the 5-HT_7 receptor gene was detected in human coronary arteries [10a]. For further characterization of the 5-HT_1-like receptor, molecular biology techniques should be utilized, indicating the presence of mRNA, pointing at certain 5-HT receptor subtypes being expressed. Functional studies, with a specifically designed spectrum of agonists and antagonists, should subsequently establish the functional relevance of this receptor.

Development of anti-migraine drugs devoid of coronary side effects

All presently available anti-migraine drugs have the potential of inducing coronary vasoconstriction [11]. Even modern anti-migraine drugs, like the selective 5-HT_{1D}-like receptor agonist, sumatriptan, may induce cardiac ischemia in some patients [12]. Attempts to develop drugs, devoid of this side-effect, are hampered by the fact that the precise mode of action of sumatriptan is not yet known in detail (see §3.4). However, two different approaches of anti-migraine drug development may be suggested to yield compounds, devoid of coronary side effects.

First, it may be speculated that the presynaptic 5-HT receptor, mediating inhibition of the release of inflammatory vasodilator peptides [13], is slightly different from the receptor mediating vasoconstriction in cranial blood vessels. Assuming that stimulation of presynaptic 5-HT receptors, and the resulting reduction of inflammatory peptide release, is sufficient to terminate the migraine-attack, compounds may be developed with affinity for the presynaptic receptor, but not for the 5-HT receptor mediating vasoconstriction. Indeed, both human and bovine cerebral arteries were shown to express mRNA for the 5-$HT_{1D\beta}$ receptor [3], whereas the expression of 5-$HT_{1D\alpha}$ receptor mRNA was shown in the human trigeminal ganglion [14]. Thus, development of a 5-$HT_{1D\alpha}$ receptor selective agonist may specifically treat the neurogenic inflammatory component of migraine, and may be devoid of effect on the putative coronary vasoconstrictor 5-$HT_{1D\beta}$ receptor [2]. However, the general validity of this concept needs confirmation: canine coronary arteries were shown to express a 5-$HT_{1D\alpha}$ receptor mRNA [4], whereas the rat trigeminal ganglion expressed mRNA for 5-HT_{1B} receptors, which is the rat homologue of the 5-$HT_{1D\beta}$ receptor [15]. The latter observations demonstrated the existence of species heterogeneity of migraine related 5-HT receptors in blood vessels and in the trigeminal ganglion, and emphasized the need for human models in anti-migraine drug development.

Secondly, it may be speculated that agonists for 5-HT receptors may behave as partial agonist in some tissues, but as a full agonist in other tissues, depending on the efficiency of the receptor-effect coupling and on the presence of a receptor reserve [16]. A 5-HT receptor agonist could then theoretically act as a full agonist in the trigeminal nerve presynaptic 5-HT receptors -thereby possibly alleviating migraine. This same compound could be only a partial agonist when causing vasoconstriction in blood vessels. However, little experimental evidence is available yet to support this hypothesis.

Lastly, it may be emphasized that very little is known about the occurrence of angina pectoris related to anti-migraine drugs in patients who usually do not belong to traditional groups-at-risk for coronary artery disease. Also the recently described large variability of the relative contribution to contraction of 5-HT_{1D}-like and 5-HT_2 receptors, has remained unexplained [2]. The potential mechanisms involved in coronary side effects of anti-migraine drugs are currently under investigation, and may provide information necessary for the development of safer anti-migraine drugs.

Are 5-HT receptor antagonists potentially effective in ischemic heart disease?

5-HT receptor antagonists have not proven very successful in the prevention of clinical symptoms in which 5-HT-induced coronary vasoconstriction was speculated to play a role. In particular, the 5-HT_2 receptor antagonist, ketanserin, was ineffective in preventing Prinzmetal's angina [17] or restenosis after PTCA [18]. However, it must be kept in mind that the patient population in the first study was rather small for definitive conclusions (five patients with Prinzmetal's angina) and that ketanserin was effective in prevention of *early* restenosis or PTCA-related vasoconstriction [19-21]. Indeed, mediators other than 5-HT appear to be responsible for chronic restenosis. Furthermore, it was pointed out in chapter 5 that low concentrations of 5-HT may exert their effects also via 5-HT_{1D}-like receptors, for which ketanserin has negligible affinity. In fact, Peroutka and Snyder showed as early as 1979 [22] that 5-HT in concentrations up to 0.1 μM hardly displaced labelling from 5-HT_2 binding sites, while 0.1 μM 5-HT completely replaced labelling from 5-HT_1 binding sites. In addition, Kaumann and colleagues (1994) [2] emphasized that, in some patients, 5-HT_1 receptors mediating contraction may predominate over 5-HT_2 receptors. Also in atherosclerosis and restenosis, in which 5-HT may act as a growth factor, the role of 5-HT_1 receptor subtypes should not yet be ruled out [23]. Lastly, 5-HT receptor antagonists may not only reduce the effect of 5-HT, but may also interfere with the amplifying interaction between 5-HT and other vasoconstrictor agonists, such as thromboxanes and perhaps endothelins (see Ref. 24, 25 and chapter 6). Interestingly, this

amplifying interaction appeared specifically linked to 5-HT_1 receptors [24, 26]. It is concluded that only the development of a mixed 5-HT_1, 5-HT_2 receptor antagonist, and its testing in clinical trials in humans, will provide a definitive answer to the question of the significance of the role of 5-HT in coronary artery disease.

Clinical prospects of endothelin receptor antagonists and converting enzyme inhibitors

Endothelin inhibitor drugs are on the verge of clinical trials and application, which is extremely early when considering that the peptide was discovered only seven years ago. Despite this, the potential areas for clinical application are still the subject of considerable debate. This paragraph will discuss these areas, and the probability of success for endothelin inhibitor drugs.

Two different approaches are currently under investigation to attenuate the effect of endothelins. First, one may inhibit the converting enzyme, which converts the precursor big-ET-1 to ET-1 by cleavage between Trp^{21} and Val^{22}. Recent advances have led to the molecular characterization of ECE-1, a membrane-bound neutral metalloprotease, which is expressed in endothelial cells throughout the body [27, 28]. This development will encourage the synthesis of more potent, and possibly more selective converting enzyme inhibitors than the presently available phosphoramidon. Secondly, one may try to develop antagonists for endothelin receptors that mediate the pathological effects. Indeed, the recent development of several non-peptide, orally active endothelin receptor antagonists, initiated by the report on Ro-462005 [29], has provided a list of interesting compounds, of which SB209670 [30], BMS182874 [31], and bosentan [32] are the most promising examples revealed thus far.

1. Hypertension. Although hypertension was among the first pathological states, in which ET-1 was believed to play a potential role [33], it was recently observed that mice partially deficient for endothelin, were slightly hypertensive, whereas hypotensiveness had been anticipated [34]. On the other hand, it has been shown that endothelin receptor antagonists may be effective in animal models of hypertension. Indeed, a sustained, dose-dependent reduction in blood pressure (-40 mm Hg) could be obtained in conscious spontaneously hypertensive and deoxycorticosterone acetate (DOCA)-salt rats following enteric administration of SB209670 or BMS182874, and in conscious rats with chronic heart failure following administration of bosentan [36] (overview, see Ref. 35). Bosentan was shown to exert additive effects when co-administered with the angiotensin-converting enzyme inhibitor cilazepril [36].

2. Pulmonary hypertension. In patients with pulmonary hypertension, a significantly increased concentration of plasma immunoreactive ET-1 has been observed. Moreover, the expression of ET-1 mRNA was augmented, which would suggest that local production of ET-1 plays a role in the characteristic vascular dysfunction and abnormal smooth muscle cell proliferation associated with pulmonary hypertension [37]. Recently, Goerre and colleagues observed increased plasma ET-1 levels in mountaineers at high altitude, possibly associated with the degree of hypoxia-induced acute pulmonary hypertension [37a].

3. Myocardial ischemia and infarction. Levels of endothelin may be increased in patients after acute myocardial infarction [38] or during angina in patients in whom acetylcholine or ergonovine had been shown to provoke coronary vasospasm [39]. ET-1 is a potent and efficacious constrictor of human coronary arteries, and some reports suggest that threshold concentrations of ET-1 could potentiate the contractile response to 5-HT [25], although this mechanism is still uncertain for the human coronary artery [40]. ET-1 immunoreactivity was recently shown to be significantly higher in coronary artery atherosclerotic specimens obtained from patients with unstable angina, than in specimens from patients with stable angina [41]. Moreover, it was observed that plasma endothelin concentrations, measured after myocardial infarction, are strongly related to clinical outcome [42] and 1-year survival ($p<0.0001$) [43]. Clinical evaluation of heart failure and atrial natriuretic factor (ANF) level, both considered predictors of mortality after myocardial infarction, provided no additional prognostic information after endothelin had been introduced into the analysis [43].

4. Restenosis and atherosclerosis. Endothelins have been implicated in restenosis and atherosclerosis. Indeed, ET-1 may induce smooth muscle mitogenesis *in vitro*, which was attenuated by the ET_A receptor antagonist BQ-123 [44], and ET-1 was shown to release a diversity of other growth factors like PDGF, and transforming growth factor-ß (see Ref. 45). Increased circulating endothelin concentrations, and augmented tissue endothelin levels were observed in patients with advanced atherosclerosis [46]. Levels of ET-1 were also elevated in patients after undergoing PTCA [47, 48], and the novel nonpeptide endothelin receptor antagonist SB209670 was recently shown to reduce neointima formation by 50% in a rat model of carotid artery balloon angioplasty [49].

5. Cerebral vasospasm following subarachnoid haemorrhage. Subarachnoid haemorrhage (SAH) is followed by cerebral vasospasm in many patients. Plasma and cerobrospinal fluid (CSF) endothelin levels were elevated in patients with SAH [50], and plasma levels of endothelin coincided with onset of vasospasm [51]. Interestingly, the administration of the ET_A receptor antagonist BQ-123 prevented development of vasospasm in a SAH model in dogs [52]. Bosentan and SB209670 were reported to exert similar protective effects [35].

6. Renal disease. Endothelins may play a role in ischemia-induced acute renal failure. Indeed, BQ-123 protected the rat kidney against ischemia-induced acute renal failure and tubular cell injury [53], and Ro462005 reduced renal vasoconstriction following reperfusion [29]. SB209670 attenuated the ischemia-induced reduction in glomerular filtration rate, and reversed the increases in fractional sodium excretion in both rats [54] and dogs [55]. BQ-123 and SB209670 were also shown to be effective in preventing acute reduction in renal blood flow and glomerular filtration rate after intravenous administration of cyclosporine A [56, 57]. The latter may be important because of the nephrotoxic effects of cyclosporin A, in which endothelins are believed to play an important role [58, 59].

7. Other targets. Additional therapeutic targets for endothelin inhibitor drugs have been suggested. There is some indication for involvement of endothelins in bone remodelling, benign prostatic hypertrophy, and hypertension associated with hemangioendothelioma [35, 60, 61].

Despite these often indirect signs of involvement of endothelins in disease, it is obvious that only clinical trials in humans will provide definitive answers with respect to the therapeutic potential of endothelin inhibitors. Recently, Vanhoutte appropriately argued that disease-related increased endothelin levels could merely represent 'a last cry for help of the dying cell mistreated by disease (or the investigator?)' [62]. Whether increased endothelin concentrations reflect an active role in pathophysiology, still remains to be established. It may also be recalled that, in rats, angiotensin-converting-enzyme (ACE) inhibitors were extremely promising in the prevention of intimal hyperproliferation after balloon angioplasty [63]. In man, however, ACE-inhibitors were not successful in the prevention of restenosis after percutaneous transluminal angioplasty (PTCA) [64]. Furthermore it can not be emphasized enough that many questions still exist about the exact nature of the receptors involved in endothelin-induced effects (Chapter 10).

Knowledge of these receptors is essential for the rational development of receptor antagonists, and could prevent disappointing clinical results with receptor antagonists, inaccurately questioning the pathophysiological role of endothelins. The novel, orally active, potent endothelin receptor inhibitors, and the ongoing development of ECE inhibitors are essential in providing an indication about the therapeutic potential of endothelin inhibitors in relevant clinical trials in man. In addition, the increasing knowledge about the role of endothelins may provide a better understanding about other diseases: Hirschsprung's disease is characterized by the congenital absence of ganglion cells in myenteric and submucosal plexus of the distal gastrointestinal tract. Recently, it was shown that this disease is closely associated with a missense mutation of the ET_B receptor gene, which may provide novel insight in mechanisms underlying this disorder [64a].

12.3 Limitations of the applied methods

Apart from the limitations to any *in vitro* model, the studies described in previous chapters highlighted two specific limitations of organ bath studies applying human blood vessels. First, the use of ring segments suspended on stainless steel tissue hooks, implies that drugs added to the organ bath, stimulate both intra-luminal (endothelial) and extraluminal (smooth muscle) cells. When receptors for the agonist under investigation are present on both endothelial and smooth muscle cells, it can be inferred that pathology-related alteration of the response mediated by receptors on the endothelium, may remain unnoticed when studied together with the more pronounced, but unaltered response mediated by receptors on the smooth muscle. For instance, 5-HT may induce relaxation, when administered in undiseased human coronary arteries *in vivo*. In patients with coronary artery disease, this response is reversed to contraction, probably because of loss of 5-HT receptor-mediated responses within the endothelium [65]. In human coronary arteries *in vitro*, it is virtually impossible to demonstrate endothelium-dependent relaxation mediated by 5-HT [6, 66]. Only contractile responses can be recorded, in healthy as well as in diseased coronary arteries. This may point at the possibility that, in the organ bath, smooth muscle-mediated responses are by far more important than those mediated by the endothelium. Therefore, altered endothelial function, as could occur in coronary artery disease, may not be revealed in the present set-up.

Secondly, it may be argued that a time span of up to 24 hour after cardiectomy, before the tissue is investigated, is too long to be able to study coronary artery reactivity. However, it should be recalled that the hearts, also used for homograft cardiac valve transplantation, are removed from brain-dead heart beating organ donors and immediately stored in a sterile, organ protecting solution, which even allows us to study mechanisms of myocardial contractility after 24 hours [67]. Also endothelial function (quantified as relaxation to 1 nM substance P) is maintained comparable to that seen in coronary arteries obtained from patients undergoing cardiac transplantation for reasons of idiopathic cardiomyopathy, and investigated immediately after explantation [68, 69]. Thus, it appears reasonable to assume that the tissue, used in our experiments, is at least as useful as coronary arteries studied by groups, exclusively investigating coronary arteries obtained from heart transplantation programs [2, 24, 70]. Interestingly, coronary arteries in our laboratory do not commonly exhibit the spontaneous phasic contractions, as has been described to hamper pharmacological analysis by many investigators. Spontaneous oscillations tend to occur more frequently in hearts obtained from diseased (transplantation) or older patients [71, 72]. Recently, it was suggested that tonic contractile responses can be studied accurately only when the calcium antagonist nifedipine (0.1 μM) is present to block oscillations [70]. In our studies it is not necessary to add such potential interference with the mechanisms under investigation, since spontaneous oscillations occur in only a very small proportion of the experiments.

12.4 Implications for future research

Receptor characterization

Receptor characterization is based on operational, transductional, and structural criteria [10, 73]. Studies assessing the receptors mediating an effect in human blood vessels should ideally consist of all three components. More specifically, attempts must be made to perform both organ bath and receptor binding experiments. Confirmation may be obtained by studying involvement of certain second messenger mechanisms. In addition to that, attempts must be made to reveal receptor mRNA encoding certain receptors, which may supply reciprocal information for further experiments. When the ligand affinity pattern of this receptor is known, this may be used to choose conclusive ligands when designing further operational experiments. Molecular biology, and radioligand binding experiments should always be accompanied by functional organ bath studies, since isolated

information on the expression of a receptor mRNA and on the presence of certain binding sites, provides no information on their involvement in a particular functional response. It should also be recalled that radioligand binding assays, second messenger assays, and molecular biology techniques usually require only little tissue, which may be an important advantage when considering the use of human tissues. In summary, now that drugs can be designed on a rational basis, attempts should be made to supply a full characterization of the receptor mediating a certain effect.

Interaction of different receptor-mediated effects

Agonists at different receptors may be involved in an amplifying interaction, which exceeds the additive effects of these compounds. Little is understood about the mechanism involved. Interference with interaction may be just as efficient as counteraction of the individual effects, and further studies may be aimed at elucidation of this phenomenon.

Development of more accurate models

Organ bath *in vitro* techniques as used in the present studies, provide a robust method which allows for investigation of multiple parallel coronary artery segments. However, as mentioned above, the present method does not allow for detailed analysis of some effects mediated via the endothelium (e.g. effects induced by 5-HT and acetylcholine). A perfused isolated coronary artery model may provide a method in which the luminal and extra-luminal side may be stimulated separately, and in which the effect of atherosclerotic changes of the coronary artery appear more likely to result in altered responses to certain pharmacological stimuli, as has also been observed in the *in vivo* situation. In addition to that, such a model would allow for *in vitro* analysis of the acute effects induced by intravascular manipulation, such as PTCA, stenting and intravascular echo.

12.5 References

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Chapter 13

Samenvatting in het Nederlands; Summary in Dutch

Hoofdstuk 1 geeft een overzicht van klinische aspecten van ischemische hartziekten. Ischemische hartziekten kunnen worden onderverdeeld in stabiele angina pectoris, instabiele angina pectoris, acuut myocard infarct, en variant angina pectoris. Deze ziektebeelden zijn onderdeel van een spectrum van gerelateerde afwijkingen, waarin de zuurstofvoorziening de behoefte niet adequaat dekt. Dit resulteert gewoonlijk in pijn op de borst (angina), en een aantal andere lichamelijke, chemische, en electrofysiologische symptomen. Bij de pathofysiologie van ischemische hartziekten is ten eerste een *passieve* obstructie van het vaatlumen betrokken. Deze ontstaat door thrombusvorming en door de vorming van een atherosclerotische plaque. Ten tweede moet worden gedacht aan *actieve* constrictie van de vaatwand door een disbalans van constrictie- en relaxatiemechanismen (de vasculaire tonus). De relatieve bijdrage van de actieve vasoconstrictie varieert van zeer gering, in geval van stabiele angina, tot vrijwel volledig, in geval van variant angina pectoris. In een enkel geval kan angina ook ontstaan als bijwerking van geneesmiddelen, zoals anti-migraine middelen. Behandeling van ischemische hartziekten is gericht op vermindering van de ischemie van het myocard, de gerelateerde angineuze pijn, en op het voorkomen van hemodynamische complicaties. Grootschalige klinische trials worden met regelmaat opgezet om de beste manier van behandeling te bepalen voor verschillende patientgroepen.

Hoofdstuk 2 beschrijft de fysiologie en pathofysiologie van vasculaire tonus. Vasculaire tonus is gedefinieerd als het evenwicht tussen relaxerende en contraherende mechanismen in de vaatwand. Verstoring van dit evenwicht zou een belangrijke rol kunnen spelen in bovengenoemde klinische syndromen van ischemische hartziekten. Vasculaire tonus is het resultaat van processen in het vasculaire gladde spierweefsel en het vasculaire endotheel. Contractie en relaxatie van glad spierweefsel ontstaan door interactie van actine en myosine eiwitketens. De interactie wordt gereguleerd door een groot aantal receptoren, enzymen, ion-kanalen, ion-pompen, en door ion-uitwisseling. Hiermee wordt de concentratie van calcium in het cytosol, of de gevoeligheid van het contractiele apparaat beïnvloed. Het endotheel heeft invloed op de vasculaire tonus door de afgifte van zowel relaxerende (endotheel afhankelijke relaxerende factor, EDRF), als contraherende stimuli

(endotheel afhankelijke contraherende factor, EDCF), die beiden een effect hebben op het onderliggende gladde spierweefsel. Verstoring van het evenwicht tussen relaxatie en contractie kan leiden tot verlaging van de doorbloeding, met daaropvolgende vermindering van de zuurstofvoorziening aan het myocard. Verstoring van dit evenwicht treedt met name op in atherosclerotische bloedvaten, waarin de endotheel-gemediëerde dilatatoire component is verminderd. Ook endotheelschade na ballon angioplastie, serum lipoproteïnen, en serum cholesterol, kunnen betrokken zijn bij verstoring van het evenwicht tussen contractie en relaxatie.

Hoofdstuk 3 geeft een overzicht van serotonine (5-HT), de serotonine receptor subtypen, en de functionele en therapeutische relevantie in het cardiovasculaire systeem. Ook wordt de ontwikkeling geschetst van de oude 'M'- en 'D'-receptorclassificatie tot aan de huidige IUPHAR classificatie voor serotonine receptoren. Hoewel exogeen toegediend serotonine een evident effect heeft op het cardiovasculaire systeem, is dit van receptor antagonisten tegen eventuele endogene serotonine vrijzetting nog niet overtuigend vastgesteld. Eigenlijk is het gebruik van serotonine agonisten en antagonisten in cardiovasculaire aandoeningen tot nu toe beperkt tot de behandeling van migraine. De teleurstellende resultaten met serotonine receptor antagonisten in coronaire vaatspasmen kunnen worden verklaard door overschatting van de rol die serotonine speelt in het ziekteproces, of door het gebruik van een receptor antagonist die gericht is op de verkeerde receptor.

In **Hoofdstuk 4** wordt beschreven hoe de receptoren werden onderzocht die de contractiele effecten mediëren van serotonine op de vena saphena van de mens. Daarbij werd gebruik gemaakt van verschillende serotonine receptor agonisten en antagonisten. De potentie volgorde van de agonisten was 5-CT ≈ 5-HT > methysergide ≈ sumatriptan ≈ α-methyl-5-HT ≈ RU 24969 ≈ DOI > 2-methyl-5-HT > 8-OH-DPAT. De 5-HT_2 receptor antagonist, ketanserine, veroorzaakte een rechtsverschuiving van met name het bovenste deel van de concentratie-effect-curve van serotonine en 5-CT, terwijl de curve van de selectieve 5-HT_2 receptor agonisten, α-methyl-5-HT en DOI, op parallelle wijze naar rechts werd verplaatst door ketanserine. Verder was de contractie door serotonine gevoelig voor antagonisme met methysergide, methiothepin, ICS 205-930 (tropisetron), en flesinoxan. De contractie door de selectieve 5-HT_1 receptor agonist, sumatriptan, kon worden geantagoneerd met methiothepin, metergoline, rauwolscine, en cyanopindolol (allen antagonist van o.m. 5-HT_1 receptor subtypen), maar niet met ketanserine. Tussen de functioneel geregistreerde pD_2 / pK_B waarden en de eerder beschreven affiniteit voor

5-HT_{1D} receptoren in bindingsstudies in hersenmembranen, werd een goede correlatie gevonden. Contracties van de vena saphena van de mens kunnen dus gemediëerd worden door zowel de 5-HT_2 receptor, alsook door een receptor die lijkt op de 5-HT_{1D} receptor die in hersenmembranen wordt gevonden.

Van drie patienten die een harttransplantatie ondergingen nadat zij 6-11 jaar eerder een coronaire bypass operatie hadden ondergaan, werd de vena saphena van het geëxplanteerde hart verwijderd. Hoewel lichtmicroscopisch enorme intimahyperplasie zichtbaar was, en hoewel de contractie op 30 mM kalium significant lager was, bleven zowel pD_2 als E_{MAX} (wanneer berekend als percentage van kalium-geïnduceerde contractie) onveranderd in geval van serotonine en sumatriptan, wanneer dat werd vergeleken met de respons van venen die direct uit het onderbeen waren verwijderd.

In **Hoofdstuk 5** wordt een serie experimenten beschreven die zijn gericht op de contractiele eigenschappen van de coronairarterie van de mens in respons op serotonine, geactiveerde bloedplaatjes, sumatriptan, en ergotamine. Ketanserine (1 μM) remde zowel de contractiele respons op serotonine als de respons op plaatjes. In aanwezigheid van ketanserine gaven rauwolscine en cyanopindolol concentratie afhankelijke additionele remming van de overgebleven respons op lage concentraties (i.e. ≤1 μM) serotonine. De respons op sumatriptan werd op gelijke wijze geantagoneerd door rauwolscine en cyanopindolol en ook door metergoline, maar niet door ketanserine. De potentievolgorde van deze antagonisten voor sumatriptan lijkt op de volgorde zoals beschreven voor de 5-HT_{1D}-achtige receptor in de humane vena saphena (Hoofdstuk 4). Tegen contracties door plaatjes was slechts een niet significant additioneel effect van rauwolscine en cyanopindolol waarneembaar. De conclusie is dat 5-HT_1 receptoren een rol spelen bij contracties door serotonine in vooral lage concentraties. 5-HT_2 receptoren spelen een belangrijke rol, met name bij hogere concentraties serotonine.

Verder werd gekeken naar de contractiele effecten van de anti-migraine middelen, sumatriptan en ergotamine, op de coronairarterie van de mens. Ergotamine was ongeveer honderd maal potenter en had een twee maal zo hoog maximaal effect.

Hoofdstuk 6 beschrijft de effecten van humane bloedplaatjes (thrombocyten) op de coronairarterie van de mens. Plaatjes veroorzaakten concentratie afhankelijke contracties van dit bloedvat. De contractiele respons leek enigszins verminderd in geval van vaatsegmenten met histologische tekenen van vroege atherosclerose. De contractie verminderde ook wanneer het bloedvat was voorbehandeld met ketanserine of met de thromboxaan receptor antagonist, SQ30741. Plaatjes, verkregen van donoren die gedurende

7-13 dagen 40 mg/dag aspirine hadden geslikt, veroorzaakten een significant lagere contractiele respons, die gepaard ging met een vrijwel selectieve verlaging van de in het orgaanbad gemeten thromboxaan concentratie. De hoeveelheid in het bad afgegeven serotonine bleef onveranderd, wat een goede verklaring lijkt voor het feit dat ketanserine de overgebleven respons van plaatjes van met aspirine behandelde plaatjesdonoren remde, terwijl de thromboxaanreceptor antagonist SQ30741 slechts geringe, niet significante additionele remming van contractie teweeg bracht. De resultaten van deze experimenten suggereren dat additioneel antagonisme van contractiele 5-HT receptoren van de coronair arterie, de klinische effectiviteit van aspirine zou kunnen verbeteren.

In **Hoofdstuk 7** wordt uiteengezet hoe endotheline (ET) receptoren in de coronair arterie, en in het ventrikel en het atrium van het humane hart zijn bestudeerd. Bij deze experimenten werd gebruik gemaakt van kwantitatieve receptor autoradiografie. De verdringing werd bestudeerd van labeling met [^{125}I]sarafotoxin S6b (30 pM) en [^{125}I]ET-1 (30 pM). Als verdringings liganden werden gebruikt de ET_A receptor selectieve ligande BQ-123, en de ET_B receptor selectieve ligande [Ala^{1,3,11,15}]ET-1. De specifieke binding was hoger in het atrium en in de coronair arterie dan in het ventrikel. In de coronair arterie was de binding het grootst in de media. ET-1 verdrong binding van [^{125}I]ET-1 en [^{125}I]sarafotoxin S6b op monofasische wijze in het atrium, in het ventrikel en in de coronair arterie. [Ala^{1,3,11,15}]ET-1 en BQ-123 verdrongen de labeling met [^{125}I]ET-1 en [^{125}I]sarafotoxin S6b op een bifasische manier in zowel het ventrikel als in het atrium. In de coronair arterie van de mens, verdrongen [Ala^{1,3,11,15}]ET-1 en BQ-123 de binding met [^{125}I]ET-1 op een monofasische manier (ET-1 > BQ-123 > [Ala^{1,3,11,15}]ET-1). Daarentegen verdrongen [Ala^{1,3,11,15}]ET-1 en BQ-123 de binding met [^{125}I]sarafotoxin S6b op bifasische wijze. De lage affiniteits-bindingsplaats van [Ala^{1,3,11,15}]ET-1 en BQ-123 correspondeerde met de bindingsplaats van [^{125}I]ET-1. Hieruit vogt dat zowel [^{125}I]ET-1 als [^{125}I]sarafotoxin S6b ET_A en ET_B bindings plaatsen in het atrium en ventrikel bezetten. In de coronair arterie bezetten beide radioliganden ET_A bindingsplaatsen, maar [^{125}I]sarafotoxin S6b bezette ook een non-ET_A, non-ET_B bindingsplaats met relatief hoge affiniteit voor BQ-123 en voor [Ala^{1,3,11,15}]ET-1.

Met in gedachten de observaties in de receptor bindingsstudies van Hoofdstuk 7, wordt in **Hoofdstuk 8** gezocht naar functioneel bewijs van overeenkomstige receptor heterogeniteit. Daartoe werden de effecten van de ET_A receptor antagonist onderzocht op contracties van de vena saphena van de mens, veroorzaakt door ET-1 of sarafotoxin S6b. Voorbehandeling met BQ-123 (0.1-1 μM) liet contractie door ET-1 onveranderd, maar

antagoneerde contractie door sarafotoxin S6b op concentratie afhankelijke wijze. Dit antagonisme was bifasisch, i.e. BQ-123 was met name actief ten opzichte van de hogere concentraties van sarafotoxin S6b. Hieruit volgt dat de contracties door ET-1 worden gemediëerd door een BQ-123-ongevoelige receptor, terwijl de contracties door sarafotoxin S6b ten dele worden gemediëerd door een receptor die verschilt van de receptor die verantwoordelijk is voor de respons door ET-1.

In **Hoofdstuk 9** worden endotheline receptoren onderzocht die contractie mediëren van de coronair arterie van de mens. Dit werd gedaan met behulp van verschillende agonisten en antagonisten voor de endotheline receptor. De potentie volgorde (pD_2) van de agonisten was: ET-1 ≈ sarafotoxin S6b > big-ET-1 ≈ ET-3. [$Ala^{1,3,11,15}$]ET-1 (een ET_B receptor agonist) had alleen effect in een concentratie van 1 μM. 0.3 μM big-ET-3 had geen effect. Verwijdering van het endotheel veranderde niets aan de contractiele respons van ET-1 en sarafotoxin S6b. Na afloop van een volledige concentratie-effect-curve met ET-1 of met sarafotoxin S6b, kon uitsluitend in de segmenten die waren blootgesteld aan sarafotoxin S6b een additionele respons worden geïnduceerd met ET-1. Andersom was dit niet mogelijk. 0.1 μM BQ-123 veroorzaakte rechtsverschuiving van de concentratie-effect-curve van sarafotoxin S6b (pK_B: 7.86). Daarentegen gaf uitsluitend een concentratie van 10 μM BQ-123 een rechtsverschuiving van de concentratie-effect-curve van ET-1 (pK_B: 5.75). Ook de ET_A receptor antagonist FR139317 was potenter ten opzichte van contracties geïnduceerd door sarafotoxin S6b dan ten opzichte van contracties geïnduceerd door ET-1. [$Ala^{1,3,11,15}$]ET-1 (1 μM) had geen effect op de concentratie-effect-curve van ET-1 of sarafotoxin S6b. Vergeleken met ringsegmenten met intact endotheel, was de pD_2 in stripsegmenten met intact endotheel significant toegenomen. In strip segmenten zorgde 1 μM BQ-123 wel voor significante rechtsverschuiving van de concentratie-effect curve van ET-1. De contractiele respons na stimulatie met big-ET-1 in endotheel-intacte coronair arterie segmenten was significant verminderd na voorbehandeling van het segment met 100 μM phosphoramidon. Dit duidde op conversie van big-ET-1 naar ET-1 in het coronair arterie segment. Hieruit volgt dat ET-1 en sarafotoxin S6b de geïsoleerde coronair arterie van de mens contraheren via verschillende receptoren, die waarschijnlijk het best kunnen worden gekarakteriseerd als subtypen van de ET_A receptor. Voorts bleek dat de manier van prepareren (ring of strip) de effectiviteit kan beïnvloeden van ET-1 als agonist, en BQ-123 als antagonist.

In **Hoofdstuk 10** wordt een overzicht gegeven van verschillende 'typische' en 'atypische' endotheline receptor mechanismen, en worden momenteel bekende experimentele bevindingen vergeleken met de officiële IUPHAR classificatie voor endotheline receptoren. Aangetoond werd dat de huidige criteria niet voldoende zijn om alle endotheline receptoren te classificeren. Er werd nadruk gelegd op strenge classificatie criteria. Deze criteria omvatten operationele en structurele aspecten, alsmede de manier van receptor transductie. Hoewel experimenteel voldoende is aangetoond dat de huidige classificatie zal moeten worden uitgebreid, en hoewel de huidige classificatie soms zelfs misleidend kan zijn, werd duidelijk gemaakt dat er nog niet voldoende experimentele aanwijzingen zijn om te komen tot een definitieve, nieuwe receptor classificatie.

In **Hoofdstuk 11** worden de effecten beschreven van nonpeptide receptor antagonisten op de contractie geïnduceerd door arginine vasopressine (AVP) in de coronair arterie van de mens. Contractie door AVP werd geantagoneerd door zowel de gepostuleerde V_1 receptor antagonist, SR 49059, als door de veronderstelde V_2 receptor antagonist, OPC-31260 (pA_2 respectievelijk 9.76 en 7.31). In tegenstelling tot de beschreven hoge affiniteit voor V_1 receptoren van de rat, was 3 μM OPC-21268 nodig om de respons op AVP te antagoneren (pA_2: 5.6). De potentievolgorde van de antagonisten (SR 49059 > OPC-31260 > OPC-21268) correspondeert met de beschreven affiniteitsvolgorde voor de recent gecloneerde V_1 receptor van de mens. Hieruit volgt dat de beschreven hoge affiniteit van OPC-21268 voor de V_1 receptor beperkt blijft tot de V_1 receptor van de rat, hetgeen de vraag opwerpt of we hier te maken hebben met een species afhankelijk verschil van dezelfde receptor, of met twee verschillende receptor (sub)typen.

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About the author

Willem Bax was born in Utrecht, The Netherlands, on 30 December 1965. After attending the Municipal Grammar School 'Johan van Oldenbarnevelt' in Amersfoort (1978-1984), he studied medicine at the Erasmus University Rotterdam, where the certificate of Medicine (drs/M.Sc.) was obtained in 1990. During his studies, he worked as a Junior Research Assistant at the Department of Oral and Maxillofacial Surgery of the University Hospital Rotterdam/Dijkzigt (Head of Department: Dr. K. de Man). In 1989, he was a trainee at Sandoz Pharma Ltd. in Basel, Switzerland (Head of Department: Dr. J.R. Fozard). After being a trainee at Duphar b.v., Weesp, The Netherlands (Head of Department: Prof. dr. B. Olivier), he joined the Department of Pharmacology of the Erasmus University Rotterdam in September 1990. Under the guidance of Prof. dr. P.R. Saxena, he worked on a project entitled 'Pharmacology of the human isolated coronary artery; effects of 5-HT, platelets, and peptides'. During the project, he was a guest in the laboratory of Dr. D. Hoyer (Sandoz Pharma Ltd., Basel, Switzerland) where the work on endothelin receptors in the human heart was initiated.

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List of abbreviations

5-HT	5-hydroxytryptamine
ACE	angiotensin converting enzyme
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
ANF	atrial natriuretic factor
ANOVA	analysis of variance
Asn	asparagine
Asp	aspartate
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
CADS	coronary artery disease scale
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic GMP
CGRP	calcitonin gene-related peptide
CK	creatine kinase
CNS	central nervous system
CSF	cerebrospinal fluid
Cys	cysteine
d	day
Da	Dalton
DAG	diacylglycerol
DHE	dihydroergotamine
DMSO	dimethylsulphoxide
DOCA	deoxycorticosterone acetate
EC_{50}	concentration of an agonist eliciting half the maximal effect
ECE(-1)	endothelin converting enzyme(-1)
ECG	electrocardiography
EDCF	endothelium-derived constricting factor

EDRF	endothelium-derived relaxing factor
EDTA	ethylene diamine tetra-acetic acid
E_{MAX}	maximal effect
ET	endothelin
g	unit of acceleration of gravity
g	gram
GDP	guanosine diphosphate
GI	gastro-intestinal
Gln	glutamine
Gly	glycine
GMP	guanosine monophosphate
GTP	guanosine triphosphate
H	histamine
HDL	high density lipoproteins
HPLC	high-performance liquid chromatography
HTK	histidine tryptophan keto-glutarate
Hz	herz
icv	intra cerebro ventricular
iv	intravenous
IC_{50}	where an agonist causes an inhibitory response, the IC_{50} is the molar concentration which produces 50% of its maximum possible inhibition.
InsP	inositol phosphates
IP_3	inositol triphosphate
K_B	the equilibrium dissociation constant (mol/litre) for a competitive antagonist
K_D	the dissociation constant for a radiolabelled drug determined by saturation analysis
K_I	the concentration of competing ligand in a competition assay which would occupy 50% of the receptors if no radioligand were present
LC	myosin light chain subunit
LDH	lactaste dehydrogenase
LDL	low density lipoproteins
Leu	leucine
m/µl	milli/micro liter
M	molar

MANOVA	multiple analysis of variance
min	minutes
MLCK	myosin light chain kinase
mm	millimeter
mRNA	messenger RNA
MW	molecular weight
N	newton
NC	not calculated
ND	not determined
NO	nitric oxide
ox-LDL	oxidized low density lipoproteins
pA_2	the negative logarithm of the concentration of antagonist which would produce a 2-fold shift in the concentration-response curve for an agonist
pD_2	the negative logarithm of the EC_{50} or IC_{50} value
PDGF	platelet-derived growth factor
PG	prostaglandin
Phe	phenylalanine
PIP_2	phosphatidyl-inositol 4,5-biphosphate
Pro	proline
PTCA	percutaneous transluminal coronary angioplasty
rod	relative optical density
rt-PA	recombinant tissue type plasminogen activator
SAH	subarachnoid haemorrhage
Sf6b	sarafotoxin S6b
SGOT	serum glutamic oxaloacetic transaminase
SIN-1	3-morpholinosydnonimine-N-ethylcarbamide
Trp	tryptophan
Tx	thromboxane
Tyr	tyrosine
UW	University of Wisconsin
V	vasopressin
Val	valine
vs	versus