In Vivo Regulation of Cardiac β Adrenoceptors by a Partial Agonist

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Thesis submitted for the degree of Doctor of Medicine at the University of Leicester

1993

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INDEX

ACKNOWLEDGEMENTS

I would like to thank Professor D B Barnett for his support and advice whilst supervising the work presented in this thesis. I would also like to thank all the staff of the Department of Pharmacology at Leicester Royal Infirmary and in particular Rajendra Mistry for his technical help in the preparation of [^{125}I]iodopindolol and with some of the adenylate cyclase assays.

Like all clinical studies, this thesis would not have been possible without the assistance of a large number of individuals to whom I owe a great debt of thanks. Foremost, I would like to thank the 55 patients who kindly agreed to participate in the study. In addition I would like to thank the staff of Groby Road Hospital for their kind cooperation ; the cardiologists Professor D P de Bono, Dr P J B Hubner, Dr A H Gershlick and Dr J D Skehan for allowing me to study their patients ; the surgeons Mr J S Bailey, Mr R K Firmin, Mr J N Leverment and Mr T J Spyt for providing me with the right atrial biopsies ; the radiologists Dr C Reek and Dr N M Hudson for reporting the coronary angiograms and MUGA scans ; Hazel Williams for performing the MUGA scans ; the physiological measurement technicians for their help with the 24 hour holter monitoring.

I am grateful to ICI Pharmaceuticals (now Zeneca Pharmaceuticals) for their financial support.

And finally I would like to thank Linda for her continuing support and encouragement.

CHAPTER 1

General introduction

1.1 Introduction

Congestive heart failure is a common disease with a high mortality. Data from the Framingham study give a prevalence in the United States of America of 0.8% between the ages of 50-59 years and 9.1% between the ages of 80-89 years. Once manifest, the mortality in men was 37% at 2 years and 83% at 6 years (Kannel and Belanger, 1991). Although the aetiology is varied, the physiological responses are similar. These include the activation of various neurohumoral systems, including the sympathetic nervous system. In addition to the potential to cause progression of the disease, increased sympathetic activity results in changes in the density and sensitivity of myocardial β adrenoceptors, which in turn may affect myocardial function. This complex inter-relation of events may be modified by drugs, particularly those that work directly on cardiac β adrenoceptors.

The introduction of the β_1 adrenoceptor partial agonist xamoterol represented a novel approach to the treatment of heart failure. The intention was to produce a drug which modulated the sympathetic stimulation of the heart. It was predicted that xamoterol would produce mild stimulation of β adrenoceptors at rest whilst limiting any adverse effects of excessive sympathetic activation at other times. This could have significant effects on the regulation of cardiac β adrenoceptors. Whilst there is limited animal data on the effect of this drug on cardiac β adrenoceptor density, there is no information on how it might affect the *in vivo* regulation of human cardiac β adrenoceptors. The research presented in this thesis was planned to investigate the effect of chronic xamoterol therapy on cardiac β adrenoceptor density and coupling to adenylate cyclase.

This chapter contains a review of the literature on sympathetic activation and β adrenoceptor regulation in heart failure, the effects of drugs acting on β adrenoceptor on this process and relevant data on xamoterol, followed by an outline of the objectives and methods of the project.

1.2 Sympathetic activation in heart failure

Clinicians have long been aware of the clinical signs of increased sympathetic tone in patients with acute heart failure - tachycardia, sweating and pallor. Many of these signs disappear as the heart failure becomes chronic. However Thomas and Marks (1978) found that plasma noradrenaline was raised in patients with chronic congestive heart failure and that the level was related to the degree of heart failure as judged by the NYHA class of the patients. Levine *et al* (1982) confirmed these findings and found a relationship between plasma noradrenaline and haemodynamic variables such as heart rate, pulmonary capillary wedge pressure and cardiac index. Not only is the plasma noradrenaline level related to the severity of the disease but it also correlates well with prognosis. Cohn *et al* (1984) followed 106 patients with moderate to severe heart failure for up to 62 months and using multivariate analysis found plasma noradrenaline to be an independent risk factor for subsequent mortality, unlike plasma renin activity, serum sodium, stroke-work index or heart rate, all of which were univariate risk factors.

Two lines of evidence demonstrate that the raised plasma noradrenaline level is the result of increased sympathetic activity (rather than decreased clearance). Studies using tritiated noradrenaline have shown increased spillover of noradrenaline from the heart and kidneys, but not the lung, in

patients with heart failure (Hasking *et al*, 1986). Although this was associated with a reduction in pulmonary and renal (but not cardiac) clearance of noradrenaline, the changes in clearance were too small to account for the increased spillover in the kidneys. Thus both the heart and the kidneys produce increased amounts of noradrenaline in chronic heart failure. Furthermore, sympathetic nervous activity, as measured by intraneural recordings in the peroneal nerve, was found to be raised in chronic heart failure and correlated with plasma noradrenaline level and left ventricular filling pressure (Leimbach *et al*, 1986).

Sympathetic activation is not the only neuroendocrine consequence of heart failure, others including changes in renin-angiotensin activity and atrial natriuretic peptide and vasopressin secretion. A recent study from the SOLVD study group (Francis *et al*, 1990) compared markers of the 4 systems in patients with left ventricular dysfunction (ejection fraction less than or equal to 30%) with and without symptoms. In the latter group, plasma noradrenaline was significantly raised whereas plasma renin activity was normal unless the patients were treated with diuretics. This suggests that sympathetic activation occurs with milder degrees of heart failure than does activation of the renin angiotensin system.

1.3 Changes in β adrenoceptors associated with heart failure

1.3.1 Changes in receptor density

Bristow and colleagues were the first to describe changes in cardiac β adrenoceptors in heart failure (Bristow *et al*, 1982). They studied left

ventricular tissue removed from patients suffering from end stage heart failure of mixed aetiology at the time of cardiac transplantation and found approximately 50% fewer β adrenoceptors when compared to similar tissue from potential donors with normal left ventricular function. This was associated with a tissue subsensitivity to catecholamines, as demonstrated by a 45% reduction in maximal isoprenaline stimulated adenylate cyclase activity and a 54 to 75% reduction in maximal isoprenaline stimulated muscle contraction. The effects of histamine (which also has an inotropic effect mediated by adenylate cyclase) on the stimulation of adenylate cyclase activity and muscle contraction were however preserved suggesting a defect at the level of the β adrenoceptor.

The reduction in tissue β adrenoceptor density (downregulation) in chronic heart failure has been confirmed in several studies. In idiopathic dilated cardiomyopathy there is evidence of selective downregulation of β_1 adrenoceptors (Bohm *et al*, 1989; Bristow *et al*, 1986; Bristow *et al*, 1989). In mitral valve disease there may be a concomitant reduction in both subtypes (Brodde *et al*, 1989). Regardless of the aetiology, the extent of the downregulation appears to be related to the severity of the disease (Bohm *et al*, 1988; Brodde *et al*, 1989; Fowler *et al*, 1986). These studies have all been performed on preparations of cell membranes and thus represent changes in surface receptors. Surface receptors are in a dynamic equilibrium with an internalised pool of receptors (Maisel *et al*, 1987). Denniss found no increase in internalised receptors in tissue from patients with heart failure suggesting that the loss in surface receptors reflected a reduction in total receptors and not just a redistribution into intracellular sites (Denniss *et al*, 1989b).

Unlike other species, there are few "spare" receptors in the human heart (Brown *et al*, 1992). This means that a maximal response only occurs at high levels of receptor occupancy. Thus a reduction in receptor density is likely to have a major effect on maximal functional response. *In vitro* studies have shown reduced inotropic responses to β adrenoceptor agonists (Bohm *et al*, 1988; Bristow *et al*, 1982; Bristow *et al*, 1986; Fowler *et al*, 1986; Neumann *et al*, 1988) and reduced β adrenoceptor agonist stimulation of adenylate cyclase (Bohm *et al*, 1988; Bristow *et al*, 1989; Denniss *et al*, 1989b). *In vivo* studies have shown progressive impairment of catecholamine mediated inotropic responses with increasing severity of heart failure, as opposed to β adrenoceptor-adenylate cyclase independent responses to calcium gluconate, digoxin and acetylstrophanthidin which are preserved (Colucci *et al*, 1988; Feldman *et al*, 1987; Fowler *et al*, 1986). That production of cAMP is deficient in heart failure is confirmed by the observation that the inotropic response to phosphodiesterase (the enzyme which metabolises cAMP) inhibition is impaired but can be potentiated by forskolin, a direct activator of adenylate cyclase (Feldman *et al*, 1987).

1.3.2 Changes in receptor coupling to adenylate cyclase

Not all the changes seen in heart failure can be explained by changes in receptor density alone. All authors agree that β_2 adrenoceptor densities are preserved in idiopathic dilated cardiomyopathy. However β_2 mediated responses are impaired despite this, suggesting a defect in coupling of receptor to adenylate cyclase. Bohm *et al* (1989) found that the β_2 adrenoceptor partial agonist dopexamine was an inotrope *in vitro* in normal heart but not in failing heart unless potentiated by the phophodiesterase inhibitor milrinone. Bristow *et al* (1989) found reduced β_2 adrenoceptor

mediated stimulation of adenylate cyclase activity in membranes from patients with end stage heart failure.

As will be described in more detail in chapter 3, β adrenoceptor stimulation of adenylate cyclase is modulated by stimulatory (Gs) and inhibitory (Gi) guanine nucleotide binding proteins (G-proteins) (Gilman, 1987). A defect at the level of the G-proteins was suggested by the observation that stimulation of adenylate cyclase activity by the non-hydrolysable GTP analogue guanylylimidodiphosphinate (Gpp(NH)p), which will activate both Gs and Gi, was reduced in membranes prepared from patients with heart failure (Bohm *et al*, 1990a; Denniss *et al*, 1989a; Denniss *et al*, 1989b; Feldman *et al*, 1988). Sodium fluoride, which only activates Gs, was as effective in heart failure membranes as normal ones (Bristow *et al*, 1982; Bristow *et al*, 1989; Feldman *et al*, 1988; Hershberger *et al*, 1991). This would suggest an isolated defect of Gi. This has been confirmed by quantitative and functional studies. Cholera toxin and pertussis toxin catalyse the ADP ribosylation of Gs and Gi respectively. If [^{32}P]NAD is used as the substrate, the respective G-proteins can be labelled with ^{32}P and quantified following gel electrophoresis. Using this technique on human ventricular tissue, several authors have observed an increase in Gi in heart failure (Bohm *et al*, 1990a; Feldman *et al*, 1988; Hershberger *et al*, 1991; Neumann *et al*, 1988). This has been confirmed using an imunoblotting technique for assaying Gi (Bohm *et al*, 1990a). The cholera toxin labelled G-protein (Gs) is not affected in man (Feldman *et al*, 1988). The cyc$^-$ variant of the S49 lymphoma cell line is deficient in Gs. It can therefore be used as an assay for Gs function. Solubilised cardiac cell membranes prepared from patients with heart failure restored adenylate cyclase activity in this cell line as effectively as those from non failing heart (Feldman *et al*, 1988).

In summary, chronic heart failure is associated with β adrenoceptor downregulation and both quantitative and qualitative defects in Gi, but not Gs. This leads to tissue subsensitivity to catecholamines. β adrenoceptor desensitisation is a well recognised consequence of agonist exposure *in vitro* (Harden, 1983) and in animal models *in vivo* (Kowalski *et al*, 1990; Tse *et al*, 1979). It is generally accepted that the changes in β adrenoceptors seen in chronic heart failure are a consequence of the increased exposure to catecholamines, especially noradrenaline, that occur as a result of sympathetic activation.

1.4 Effect of drugs acting on β adrenoceptors on receptor regulation

1.4.1 β adrenoceptor agonists

The poor availability of human cardiac tissue limits the direct study of agonist induced changes in β adrenoceptors in man. In a rat model, chronic treatment with isoprenaline causes β adrenoceptor downregulation (Kowalski *et al*, 1990; Tse *et al*, 1979) and desensitisation of both adenylate cyclase stimulation and functional responses in rat heart (Tse *et al*, 1979). Chronic noradrenaline infusion in dogs does not produce β adrenoceptor downregulation but does desensitise inotropic responses to isoprenaline *in vivo* and adenylate cyclase stimulation *in vitro* (Vatner *et al*, 1989).

Perhaps the most direct evidence for agonist induced changes in human cardiac β adrenoceptors is the downregulation and desensitisation observed in association with sympathetic activation in heart failure as described above.

The partial reversal of the downregulation by β adrenoceptor antagonists described below supports the hypothesis that the changes in receptors are due to catecholamine exposure. Further indirect evidence for desensitisation of β adrenoceptors by agonists can be gained from the response to exogenous agonists. Whilst acute therapy with β adrenoceptor agonists in patients with heart failure produces improvements in cardiac function, chronic therapy is associated with the rapid development of tolerance. Thus Unverferth demonstrated that the cardiac output following 4 days continuous infusion with dobutamine was reduced by 43% with reference to the 2 hour value (Unverferth *et al*, 1980). Prolonged oral therapy with both the β_1 selective agonist prenalterol and the β_2 selective agonist pirbuterol has also been associated with the development of tolerance (Colucci *et al*, 1981; Currie *et al*, 1984a; Lambertz *et al*, 1984; Roubin *et al*, 1984; Weber *et al*, 1982). In an open study of chronic pirbuterol therapy, Colucci observed that the development of tolerance to its clinical effects was temporally associated with the downregulation of lymphocyte β adrenoceptors. Lymphocytes however are poor surrogates for cardiac tissue as they express only the β_2 subtype (compared to a majority of β_1 adrenoceptors in the heart) and they are not innervated. Brodde found that 9 days therapy with the β_2 adrenoceptor partial agonist procaterol desensitised β_2 adrenoceptor responses in normal volunteers, whereas 14 days therapy with the β_1 adrenoceptor partial agonist xamoterol desensitised β_1 adrenoceptor mediated responses (Brodde *et al*, 1990). However there is some concern that the latter effects may be an artefact resulting from incomplete washout of xamoterol (see below).

1.4.2 β adrenoceptor antagonists

In 1973 Slome reported the cases of 2 patients who developed myocardial infarcts 2 and 3 days following the withdrawal of propanolol therapy (Slome, 1973). Since then there have been numerous reports of adverse cardiac events following abrupt β blocker withdrawal (Houston and Hodge, 1988). There is considerable evidence that this withdrawal syndrome is related to β adrenoceptor hypersensitivity resulting from treatment with β adrenoceptor antagonists.

Increased sensitivity to isoprenaline infusion following propanolol withdrawal has been demonstrated in normal subjects and hypertensive patients (Boudolas *et al*, 1977; Nattel *et al*, 1979). Subsequently Aarons and colleagues demonstrated that propanolol therapy increased the density of β adrenoceptors on human lymphocytes, despite some retained propanolol in their membrane preparation as indicated by a decreased affinity for the radioligand used in their receptor assay (Aarons *et al*, 1980). Receptor density remained significantly elevated for 2 days after withdrawal. That this might relate to changes in cardiac β adrenoceptor density was suggested by data from the same group using an animal model. Upregulation of lymphocyte β adrenoceptors in rats following a 7 day infusion with propanolol by subcutaneous osmotic minipumps was associated with upregulation of both β_1 and β_2 adrenoceptor subtypes in heart and lung (Aarons and Molinoff, 1982).

As mentioned above, human lymphocytes are a poor surrogate for cardiac tissue in the study of β adrenoceptor regulation. The wide acceptance of coronary artery bypass surgery for ischaemic heart disease has facilitated the study of the effect of β adrenoceptor antagonists on human cardiac tissue. β

blockers are standard therapy for such patients who can tolerate them and myocardial tissue can be obtained at the time of surgery. The most common source is the right atrial appendage which is cannulated to provide venous access for the cardio-pulmonary bypass circuit. Whilst this is not ventricular tissue, it does have the advantage of being distant from the major ischaemic areas and any myocardial fibrosis.

Using such atrial biopsies, Hedberg *et al* (1985) were the first to demonstrate β adrenoceptor upregulation in human myocardium following chronic therapy with β adrenoceptor antagonists. They found increased densities of both β adrenoceptor subtypes in patients treated with various β adrenoceptor antagonists when compared with patients undergoing cardiac surgery who were not taking such drugs. Unfortunately the majority of the patients in the control group were undergoing valve surgery and are likely to have had some degree of heart failure. However β adrenoceptor upregulation in right atrial appendages from patients taking β adrenoceptor antagonists has been confirmed by several other studies of patients undergoing coronary artery bypass surgery, although none was a prospective controlled trial. Although haemodynamic variables were well matched in two studies (Bjorneheim *et al*, 1990; Golf and Hansson, 1986) and no patients had "acute heart failure" in a further 2 studies (Michel *et al*, 1988; Motomura *et al*, 1990), there remains some concern that there were differences in the treatment and control groups that might have affected receptor densities.

In an analogous fashion to the changes described in heart failure (selective β_1 adrenoceptor downregulation and β_2 adrenoceptor uncoupling), it would appear that both β adrenoceptor density and coupling to adenylate cyclase and functional responses can be regulated in a subtype selective fashion by various β adrenoceptor antagonists. Hence Michel found that the non

selective antagonists sotalol and propanolol upregulated both β_1 and β_2 subtypes whereas the β_1 selective antagonists atenolol and metoprolol upregulated only β_1 adrenoceptors (Michel *et al*, 1988). Selective upregulation of β_1 adrenoceptors by β_1 selective antagonists was confirmed in a study by Motomura (Motomura *et al*, 1990). Pindolol, a non selective antagonist with weak β_2 partial agonist activity, selectively downregulates β_2 adrenoceptors whilst upregulating β_1 adrenoceptors (Bjorneheim *et al*, 1990; Michel *et al*, 1988). Rather curiously Golf found that pindolol was more effective at upregulating total receptors than full antagonists (Golf and Hansson, 1986). The selective downregulation of β_2 adrenoceptors by pindolol is compatible with earlier data which revealed that this drug downregulated lymphocyte β adrenoceptors (Hedberg *et al*, 1986).

Chronic therapy with β_1 adrenoceptor antagonists is associated with an enhancement of β_2 adrenoceptor function. Golf found enhanced β_2 adrenoceptor coupling to adenylate cyclase, characterised by a higher ratio of terbutaline (a β_2 partial agonist) to isoprenaline (a non selective β agonist) stimulation of adenylate cyclase, in patients pretreated with the β_1 antagonists metoprolol and atenolol compared to those not treated with a β blocker (Golf and Hansson, 1986). Motomura found greater isoprenaline stimulated adenylate cyclase activity in patients treated with the β_1 selective antagonists atenolol, metoprolol or bisoprolol than in those not treated with a β blocker. Since the majority of β adrenoceptor coupling to adenylate cyclase in humans is mediated via the β_2 adrenoceptor (Bristow *et al*, 1989; Gille *et al*, 1985; Waelbroeck *et al*, 1983), this would suggest enhanced β_2 coupling. This was supported by functional studies in which the β_2 adrenoceptor agonist procaterol was both more potent and efficacious at increasing the force of contraction in isolated myocardium following β_1 adrenoceptor blockade (Motomura *et al*, 1990).

This was confirmed by Hall and colleagues (Hall *et al*, 1990) who found that, unlike the relatively β_1 adrenoceptor selective agonist noradrenaline, the non selective agonist adrenaline was more potent at increasing contractility in strips of right atrium from patients pre-treated with the β_1 selective antagonist atenolol than those not treated with a β blocker. Furthermore the β_2 selective partial agonist salbutamol had a greater intrinsic activity in these patients. The response to dibutyryl cAMP was similar in the 2 groups suggesting that the enhancement occurred at a site between the receptor and adenylate cyclase. These *in vitro* studies were extended to an *in vivo* study. They had previously shown that the increased heart rate produced by intracoronary salbutamol was due to a direct stimulation of cardiac β_2 adrenoceptors rather than secondary to vasodilatation produced by stimulation of peripheral vascular receptors since the same dose given directly into the aortic root (and hence systemically) had no effect on heart rate (Hall *et al*, 1989). They subsequently found that the dose of intracoronary salbutamol required to raise the heart rate by 30 beats per minute was significantly less in those patients pretreated with atenolol (Hall *et al*, 1991). The mechanism for this cross regulation of pathways is not known.

1.5 β adrenoceptor regulation in the treatment of heart failure

In chronic heart failure myocardial β adrenoceptors are exposed, but are subsensitive, to increased levels of endogenous catecholamines. Prolonged therapy with β adrenoceptor agonists would be expected to produce further receptor desensitisation which may explain the development of tolerance as described above. Furthermore, it has long been recognised that

catecholamines are potentially toxic to myocardium and further agonist exposure may be dangerous. In 1906 Pearce described the histological appearances of hearts from rabbits treated with intravenous injections of adrenaline (Pearce, 1906). He noted myocardial oedema, damaged muscle fibres and, in animals that survived more than 7 days, increased fibrosis. Szakacs and Cannon (1958) reported a similar picture of oedema, myofibrillar damage and leucocyte infiltration in 2 patients who had died after prolonged therapy with noradrenaline for shock and noted a similarity to the cardiac lesions seen in patients who had died with phaeochromocytomas and dogs infused with noradrenaline. Van Vliet *et al* (1966) retrospectively reviewed the autopsy findings of 26 patients who had died with phaeochromocytomas and found that 15 (58%) had an appearance which they termed "active catecholamine myocarditis" with oedema, degeneration and necrosis of myocardial fibres and fibrosis. Noradrenaline exposure is toxic to isolated feline myocytes and the mechanism appears to be a β adrenoceptor mediated calcium overload (Mann *et al*, 1992). It should be stressed that in experimental models very high concentrations of catecholamines were used. However even therapeutic concentrations may be potentially toxic. In a placebo controlled trial of intravenous dobutamine in the treatment of heart failure, given intermittently to avoid tacchyphylaxis, there was a trend for increased mortality in the treatment arm (Dies *et al*, 1986). Furthermore chronic therapy with phosphodiesterase inhibitors, an alternative method of increasing intracellular cAMP, is associated with significantly increased mortality (Packer *et al*, 1991).

However, a therapy known to decrease neurohumoral activation in heart failure has been shown to improve mortality. Angiotensin converting enzyme (ACE) inhibition, a treatment which not only interferes with the renin angiotensin system but also reduces sympathetic activation, is associated

with improved mortality in both moderate and severe heart failure (The CONSENSUS Trial Study Group, 1987; The SOLVD Investigators, 1991). It would appear that this is not just a vasodilator effect as the reduction in mortality with ACE inhibitors is greater than that of isosorbide mononitrate and hydrallazine (Cohn *et al*, 1991). As sympathetic activation is associated with increased mortality, an alternative approach to the management of chronic heart failure might be to reduce sympathetic stimulation rather than augment it with β adrenoceptor agonists.

In 1975, long before the effect of β adrenoceptor antagonists on cardiac receptors was appreciated, Waagstein reported the cases of 7 patients with severe chronic heart failure secondary to idiopathic dilated cardiomyopathy who had improved on treatment with β adrenoceptor antagonists (Waagstein *et al*, 1975). Since then there have been numerous studies on β blocker therapy in heart failure, though many are of small numbers and uncontrolled. Most studies have used β_1 selective adrenoceptor antagonists and restricted aetiology to idiopathic dilated cardiomyopathy. In placebo controlled trials, metoprolol (Anderson *et al*, 1985; Engelmeier *et al*, 1985) and bucindolol (Gilbert *et al*, 1990) have been shown to improve symptoms and left ventricular function, but not necessarily exercise tolerance, in patients with idiopathic dilated cardiomyopathy. 2 placebo controlled trials have failed to demonstrate any improvement, but the treatment periods were shorter (4 weeks) and the aetiology of the heart failure different from the positive trials, with the majority of the patients suffering from alcoholic cardiomyopathy in one trial (Ikram and Fitzpatrick, 1981) and 40% of patients suffering from ischaemic heart disease in the other (Currie *et al*, 1984b). Varying responses of patients with heart failure of different aetiology to β adrenoceptor antagonist therapy has been shown in a controlled trial of bucindolol which produced significant symptomatic and haemodynamic improvements in

patients with idiopathic dilated cardiomyopathy but not with ischaemic cardiomyopathy, in which the only improvement seen was a minor decrease in left ventricular end diastolic dimensions (Woodley *et al*, 1991). The reason for the difference is not clear but may be due to the greater fibrosis in ischaemic heart disease (and hence less viable muscle to improve).

It is not clear whether the symptomatic and haemodynamic improvements seen will translate into improved survival. Swedberg found improved survival in a group of patients treated with either alprenolol or metoprolol when compared with a historically matched group of patients not treated with β blockers (Swedberg *et al*, 1979). In a prospective placebo controlled trial analysed by intention to treat, Anderson was unable to confirm these findings (Anderson *et al*, 1985). A major difference between the 2 studies was the mortality in the control group, which was much lower in the prospective controlled trial.

The original rationale for using β blockers in the treatment of heart failure was to limit the tachycardia. The actual mechanism for the improvement is not understood. However, 2 studies have shown significant increases in right ventricular β adrenoceptor densities following therapy with metoprolol in heart failure due to idiopathic dilated cardiomyopathy. Heilbrunn showed a doubling in density from a mean of 38 to 80 fmols/mg protein after 6 months therapy (Heilbrunn *et al*, 1989) and Waagstein a 60% increase from 30 to 49 fmols/mg protein (Waagstein *et al*, 1989). In the latter study right ventricular biopsies from potential heart donors with normal left ventricular function had a mean receptor density of 97 fmols/mg protein, suggesting that antagonist therapy did not completely correct the receptor downregulation of heart failure. It is difficult, however, to explain the improvement in function solely on changes in receptor density. The dose of antagonists used in the trials

was carefully titrated to standard therapeutic dosages, which would be expected to block the upregulated receptors. *In vivo* studies using animals and human lymphocytes show rapid upregulation of receptors by antagonists and yet the clinical benefits of β blockers in heart failure may take much longer. It is not yet known which receptor subtype is upregulated and there is no information on changes in receptor coupling in response to β blocker therapy in heart failure. If the data from patients without heart failure can be extrapolated to those with heart failure, it is possible that β_1 adrenoceptor blockade could enhance β_2 adrenoceptor function.

β adrenoceptor antagonist therapy in heart failure has yet to gain wide acceptance. They are of proven benefit only in patients with idiopathic cardiomyopathy, a relatively rare cause of heart failure, but not in ischaemic heart disease, a common cause of heart failure. Furthermore, even in these selected patients not all will tolerate β blockade, with most trials reporting patients who deteriorated on the introduction of therapy. It is not clear which drug is most effective, with no comparative trials available. Finally unlike therapy with ACE inhibitors there are as yet no large scale mortality trials. Thus whilst modulation of sympathetic activation appears an attractive approach to treating heart failure, the method of achieving it has yet to be determined.

1.6 Xamoterol

1.6.1 *In vitro* and *in vivo* properties

Xamoterol (Corwin, ICI 118587) is a β_1 selective partial agonist. When fully occupying receptors, a partial agonist will have a maximal effect less than that of a full agonist. It will compete with other receptor ligands and will behave as an antagonist to receptor stimulation by agonists with a higher intrinsic activity. Table 1.1 summarises the available data on xamoterol's *in vitro* efficacy. It can be seen that there are considerable species differences in inotropic and chronotropic responses. In man xamoterol is only a weak inotrope as it does not produce significant inotropic effects *in vitro* unless potentiated with forskolin or the phosphodiesterase inhibitor milrinone (Bohm *et al*, 1990b). This does however confirm that the inotropic effect of xamoterol is mediated by cAMP, despite the fact that it does not consistently stimulate adenylate cyclase in human myocardial cell membranes *in vitro* (Bohm *et al*, 1990b; Lemoine *et al*, 1989).

It is impossible to obtain data on intrinsic activity *in vivo* without abolishing cardiovascular responses. In vagotomised and catecholamine depleted dogs, Nuttall and Snow (1982) found that xamoterol increased heart rate by 43% of the maximal effect of isoprenaline. In 2 dogs there was a linear relationship between heart rate and contractility as measured by the rate of increase in left ventricular pressure (LV dP/dt) and it was inferred that the inotropic effect was of a similar order of magnitude to the chronotropic effect. Isoprenaline produced a β_2 adrenoceptor mediated reduction in hind limb perfusion pressure, but xamoterol had no effect thus demonstrating its β_1 selectivity as an agonist. In catecholamine depleted and vagotomised rats,

Kowalski *et al* (1990) demonstrated a chronotropic effect that was approximately 75% that of isoprenaline.

Such experiments are of course impossible to perform in human subjects. The closest study was performed by Jennings who studied normal volunteers whose cardiovascular reflexes had been blocked by a combination of prazosin, clonidine and atropine (Jennings *et al*, 1984). Under such conditions xamoterol significantly increased cardiac output by 23%, mean arterial pressure by 16% and heart rate by 28%. This was less than the effect of isoprenaline infusion, but because the highest dose of isoprenaline used did not produce a maximal increase in heart rate it was not possible to calculate the intrinsic activity of xamoterol. However this does confirm that xamoterol is a partial agonist for inotropic and chronotropic responses in the healthy human heart at rest *in vivo*. Mean arterial pressure fell following isoprenaline infusion (secondary to a β_2 mediated reduction in peripheral vascular resistance) but not following xamoterol infusion, again demonstrating the β_1 selectivity of the agonist effects of xamoterol. There have been several other studies demonstrating that xamoterol increases resting heart rate and left ventricular contractility (positive dP/dt) and relaxation (negative dP/dt) in human subjects with intact cardiovascular reflexes (table 2.2).

Table 1.1. *In vitro* studies of xamoterol. AC=adenylate cyclase

Species	Tissue	Effect	Intrinsic activity	Study
Rat	Isolated myocytes	AC stimulation	approx 30%	(Limas and Limas, 1990)
Rat	Right atrium	Rate	55%	(Malta *et al*, 1985)
Rat	Right atrium	Rate	65%	(Kowalski *et al*, 1990)
Guinea pig	Right atrium	Rate	16%	(Malta *et al*, 1985)
Guinea pig	Right atrium	Rate	35%	(Hattori *et al*, 1987)
Guinea pig	Left atrium	Force	0%	(Malta *et al*, 1985)
Guinea pig	Left atrium	Force	0% (21% in reserpine treated animals)	(Hattori *et al*, 1987)
Guinea pig	Right ventricle	Force	0%	(Malta *et al*, 1985)
Guinea pig	RV papillary muscle	Force	33%	(Hattori *et al*, 1987)
Cat	Right atrium	Rate	31%	(Malta *et al*, 1985)
Cat	Right atrium	Rate	approx 60%	(Lemoine *et al*, 1989)
Cat	Left atrium	Force	21%	(Malta *et al*, 1985)
Cat	Left atrium	Force	approx 60%	(Lemoine *et al*, 1989)

Table 1.1 (continued)

Species	Tissue	Effect	Intrinsic activity	Study
Cat	RV papillary muscle	Force	50%	(Lemoine *et al*, 1989)
Cat	Right ventricle	AC stimulation	10-20%	(Lemoine *et al*, 1989)
Rabbit	Isolated heart	Rate	27%	(Vigholt-Sorensen and Nielsen-Kudsk, 1986)
Rabbit	Isolated heart	Contraction	41%	(Vigholt-Sorensen and Nielsen-Kudsk, 1986)
Man	Left ventricle	AC stimulation	not consistently detected	(Lemoine *et al*, 1989)
Man	Left ventricle (NYHA IV heart failure)	AC stimulation	0%	(Bohm *et al*, 1990b)
Man	LV papillary muscle	Force	0% (15-30% in presence of forskolin or milrinone)	(Bohm *et al*, 1990b)
Man	Right atrium	Force	0% (20% in presence of forskolin or milrinone)	(Bohm *et al*, 1990b)

By virtue of its nature as a partial agonist, xamoterol will antagonise the effect of agonists with a stronger intrinsic activity. This was demonstrated by Nuttall & Snow in their canine model (Nuttall and Snow, 1982). Xamoterol limited the heart rate response to both exogenous and endogenous (released by sympathetic nerve stimulation in dogs not depleted of catecholamines) noradrenaline. This can also be demonstrated in human subjects by observing the alterations in heart rate response to exercise. This was perhaps most elegantly shown by Sato who correlated plasma noradrenaline levels with various haemodynamic variables on exercise both before and after intravenous xamoterol in patients with mild to moderate heart failure (Sato *et al*, 1987). Heart rate, cardiac index and systolic blood pressure were all significantly higher at rest after xamoterol. Regression lines of plasma noradrenaline against heart rate, cardiac index and systolic blood pressure were all significantly less steep after xamoterol. The lines crossed at plasma noradrenaline levels between 380 and 530 pg/ml, which was equivalent to a heart rate of approximately 100 beats per minute and between one and two thirds maximal work load. In patients with ischaemic heart disease and good left ventricular function, Detry *et al* (1984) found a linear relationship between heart rate before and after intravenous xamoterol. When the control heart rate was less than 89 beats/min it was increased by xamoterol, whereas when it was greater than 89 beats/min it was decreased. In normal volunteers, heart rate was lower following xamoterol at work loads of half maximum or greater (Jennings *et al*, 1984).

Table 2 *In vivo* haemodynamic studies of xamoterol in humans

Subjects	Effect		Study
Normal volunteers	*Rest*	- supine HR increased - systolic BP increased - CO increased 15-20%	(Jennings *et al*, 1984)
	Exercise	- HR reduced at high workloads - CO unchanged at high workloads	
Idiopathic dilated cardiomyopathy, NYHA II & III, LVEF 24-52%	*Rest*	- HR unchanged - CI unchanged - LVESVI reduced 13% - LVEDVI reduced 10% - LV (+) dP/dt increased 28%	(Thierfelder *et al*, 1991)
Idiopathic dilated cardiomyopathy NYHA II-IV	*Rest*	- HR unchanged - LV (+)dP/dt increased 47% - LVEDP decreased 30% - CI increased 11%	(Simonsen, 1984)
Heart failure (various aetiology) NYHA II-III	*Rest*	- HR increased 21% - CI increased 10% - Systolic BP increased 8% - PCWP reduced 21%	(Sato *et al*, 1987)
	Exercise	- HR reduced 8% at peak - systolic BP unchanged - CI unchanged	
Heart failure (various aetiology) NYHA II-III LVEF 11-65%	*Rest*	- HR reduced 5% - RPP reduced 8% - CI increased 12% - PCWP unchanged	(Virk *et al*, 1989)
	Exercise	- HR reduced 13% - RPP reduced 14% - CI unchanged - PCWP unchanged	
Coronary artery disease, moderate angina, LVEF 45-70%	*Rest*	- HR increased 5% - CO unchanged - PRP increased 8%	(Detry *et al*, 1984)
	Exercise	- HR reduced 14% - PRP reduced 19% - CO unchanged	

Table 2 (continued)

Subjects	Effect		Study
Coronary artery disease, moderate to severe angina, LVEF 41-61%	*Rest*	- HR increased 13% - LV systolic pressure increased 12% - LVEDP reduced - LV (+)dP/dt increased	(Ikaheimo and Takkunen, 1984)
Coronary artery disease, previous MI, LVEF 26-62%	*Rest*	- HR varied response - LVEDP unchanged - LV (+)dP/dt increased 35% - LV (-)dP/dt increased 14%	(Rousseau *et al*, 1983)
Coronary artery disease, previous MI, Moderate angina, LVEF 18-57%	*Rest* *Exercise*	- HR unchanged - LV (+)dP/dt increased 33% -LV (-)dP/dt increased 19% - HR unchanged - LV (+)dP/dt unchanged at peak - LV (-)dP/dt unchanged at peak	(de Feyter *et al*, 1990)

Abbreviations

BP	blood pressure
CI	cardiac index
CO	cardiac output
HR	heart rate
LV (+)dP/dt	peak rate of rise of left ventricular pressure
LV (-)dP/dt	peak rate of fall in left ventricular pressure
LVEDVI	left ventricular end systolic volume index
LVESVI	left ventricular end systolic volume index
LVEF	left ventricular ejection fraction
MI	myocardial infarction
PCWP	pulmonary capillary wedge pressure

These data would suggest that xamoterol is a relatively weak agonist in man, with an efficacy similar to a plasma noradrenaline level of 400-550 pg/ml and will behave as an antagonist at moderate or greater degrees of exertion. In an earlier study using the same assay, Sato had found patients with NYHA class III heart failure had plasma concentrations of noradrenaline of 467 pg/ml and class IV of 807 pg/ml. Thus it would be predicted that xamoterol would behave as an agonist at rest in NYHA class I and II heart failure but as an antagonist in NYHA class IV heart failure. Indeed in a study of the acute effects of xamoterol in patients with idiopathic dilated cardiomyopathy, the only patient with NYHA class IV heart failure deteriorated whilst those in class II and III improved haemodynamically (Simonsen, 1984).

Although xamoterol develops β adrenoceptor antagonist properties on exertion, not all haemodynamic changes are attenuated. Thus although Sato *et al* found that the relationship between plasma noradrenaline to cardiac output on exertion was altered by xamoterol therapy, the cardiac output at any given work load was not reduced. This may in part be explained by the fact that plasma noradrenaline was greater at each work load after xamoterol, a feature also observed after acute antagonist therapy (Irving *et al*, 1974). In fact, none of the haemodynamic studies has shown an adverse effect of xamoterol on cardiac output, LV contractility or LV filling pressure on exertion.

The limitation of heart rate rise on exertion would suggest that xamoterol might have a useful role in ischaemic heart disease. Detry *et al* (1984) found that the rate pressure product, an indirect measure of cardiac work, was significantly lower in patients suffering from angina pectoris at submaximal and maximal exercise following a single intravenous dose of xamoterol. This was associated with increased exercise tolerance and reduced ST segment depression at comparable workloads. A single oral dose of xamoterol has

also been shown to decrease ST depression on exercise in patients with angina and preserved left ventricular function (Barrios *et al*, 1986). One week of therapy with xamoterol at once daily doses of 200, 400 and 600 mg reduced exercise heart rate and prolonged exercise duration in patients with ischaemic heart disease, angina and left ventricular ejection fractions of less than or equal to 30% (Molajo *et al*, 1987).

Xamoterol may also have some effect on ischaemic left ventricular dysfunction. Rousseau performed left ventricular angiograms before and after intravenous xamoterol in patients with previous myocardial infarcts and showed a significant improvement in contraction of hypokinetic segments of the left ventricular wall (Rousseau *et al*, 1983). In an echocardiographic study, Ikaheimo *et al* (1984) found that whilst xamoterol improved overall left ventricular function, regional wall abnormalities remained unchanged in 5 patients, improved in 1 and deteriorated in another.

As xamoterol may increase both heart rate and systolic blood pressure at rest, it may increase resting cardiac work. Although Detry found a reduced rate pressure product on exercise in patients with coronary artery disease and normal left ventricular function, the rate pressure product at rest was significantly greater after xamoterol (Detry *et al*, 1984). This could potentially increase myocardial oxygen demand which would be disadvantageous in patients with severe ischaemic heart disease. Rousseau *et al* (1983) found no change in resting myocardial oxygen consumption in patients with ischaemic heart disease and impaired left ventricular function. They felt that the reduced wall stress and improved cardiac relaxation might have reduced myocardial oxygen demand and improved perfusion of ischaemic areas. Thierfelder *et al* (1991) similarly found no significant change in resting myocardial oxygen consumption in patients with NYHA class II or III heart

failure, though when expressed as oxygen consumption per heart beat there was a small rise after intravenous xamoterol. In vitro, xamoterol produced a small increase in myocardial oxygen consumption in isolated rabbit hearts, but limited the increase in oxygen consumption produced by isoprenaline (Vigholt-Sorensen and Nielsen-Kudsk, 1986). There is no data on the effect of xamoterol on myocardial oxygen consumption on exercise, though as it decreases the rate pressure product on exercise it would be expected to decrease oxygen consumption.

1.6.2 Clinical Trials

Xamoterol would be expected to be of most use in patients with mild to moderate heart failure (ie without marked increases in sympathetic tone) when it should behave as a weak agonist at rest and on mild exercise, but limiting excessive stimulation at greater levels of exercise.

Vigholt-Sorensen performed a double blind crossover study comparing 28 days treatment with xamoterol 200 mg bd with placebo in 21 patients with ischaemic heart disease and impaired left ventricular function (20 in NYHA class II and one in NYHA class III) (Vigholt-Sorensen *et al*, 1989). Xamoterol therapy was associated with a 9% increase in exercise duration and a 13% increase in exercise workload. Only 3 patients in the placebo phase and 2 in the xamoterol phase stopped exercising because of chest pain, the majority stopping with breathlessness. This suggests that xamoterol may have improved left ventricular function in addition to any anti-ischaemic effects. This was certainly the case at rest since resting left ventricular ejection fractions increased by 11% in the xamoterol phase. Following the double blind crossover study, 18 patients were prescribed open label xamoterol for a

period of 18 months after which a further crossover study was performed in 14 patients. Xamoterol therapy was associated with significantly greater exercise duration and workload compared with placebo after 18 months therapy suggesting that tolerance had not developed to its clinical effects (Vigholt-Sorenson and Faergeman, 1990).

The largest placebo controlled trial in this group of patients was performed in Germany and Austria (The German and Austrian Xamoterol Study Group, 1988). 433 patients were randomised to receive placebo, xamoterol 200 mg bd or digoxin 0.125 mg bd for 3 months in the ratio 1:2:1. The entry criteria were breathlessness and/or fatigue on exercise (NYHA class II or III). There was no requirement to document LV function and only 35% of those randomised to xamoterol and 39% of those randomised to placebo had cardiomegaly on chest X ray. Angina was present in 48% of the xamoterol patients and 53% of the placebo group. Xamoterol therapy was associated with a 33% improvement in exercise tolerance, significantly more than digoxin (17%) and placebo (5%). In addition the symptoms of breathlessness, tiredness, palpitations and chest pain were all significantly better on xamoterol than placebo. A smaller study of 240 patients conducted in the United Kingdom found a 19% improvement in exercise capacity following 3 months treatment with xamoterol compared to a 7% improvement with placebo which was clinically significant after adjustment for baseline differences and a "statistical outlier" (Waller *et al*, 1989).

It is possible that the improvement in exercise tolerance demonstrated in the trials was related to an anti-ischaemic effect as many of the patients had ischaemic heart disease. However an analysis of a subset of 269 patients from 3 large multicentre trials (including the 2 mentioned above) who had radiological evidence of cardiomegaly and whose exercise tolerance was

limited by breathlessness or fatigue but not angina showed that xamoterol significantly improved exercise tolerance and the symptoms of breathlessness and tiredness (Marlow, 1990).

1.6.3 Effect of xamoterol on β adrenoceptor regulation

β adrenoceptors are dynamically regulated *in vivo* by the fluctuating levels of endogenous catecholamines. From the discussion above it will be seen that drugs acting on β adrenoceptors can modulate this regulation. Hence a β adrenoceptor antagonist will competitively inhibit the endogenous catecholamines and produce receptor upregulation. Pharmacological doses of full agonists will produce a greater stimulation than physiological levels of endogenous catecholamines and are likely to cause receptor desensitisation, manifest clinically by the development of tolerance. A β adrenoceptor partial agonist such as xamoterol is therefore likely to have a complicated effect on the modulation of receptor regulation, since at low levels of sympathetic tone it behaves as a weak agonist and at high levels of sympathetic tone it behaves as an antagonist. With the exception of one study, the clinical data available to date would suggest that chronic therapy with xamoterol is not associated with the development of tolerance. This would suggest a relatively neutral effect of xamoterol on receptor regulation.

The exception was a study by Brodde *et al* (1990) in normal volunteers treated for 14 days with xamoterol 200 mg bd. The heart rate response to exercise (performed whilst on treatment) was attenuated by xamoterol and the dose response of the rise in systolic blood pressure in response to isoprenaline infusion (performed 13 to 15 hours after the last dose of xamoterol) was shifted to the right. Both effects are mediated by β_1

adrenoceptors. This was interpreted to indicate that chronic xamoterol therapy desensitised β_1 adrenoceptors. However the effects on exercise heart rate are similar to previous studies following acute therapy (Detry *et al*, 1984; Jennings *et al*, 1984; Sato *et al*, 1987) and would be expected by the antagonism of a partial agonist for a full agonist. Since the isoprenaline infusion was performed without an adequate washout, the effects on systolic blood pressure could be similarly explained.

There are only 2 direct studies of receptor regulation and xamoterol, both in rat models. Limas and Limas (1990) studied the effect of xamoterol on β adrenoceptors on myocytes isolated from rat hearts by enzymatic digestion. Xamoterol produced a reversible time and dose dependent loss of surface receptors, with a similar time course but to a lesser extent than the isoprenaline. However the effect of isoprenaline on downregulating receptors was attenuated by the presence of xamoterol, showing that xamoterol modulates the effect of a full agonist on receptor regulation.

Kowalski *et al* (1990) compared the effects of a 6 day infusion of xamoterol with that of isoprenaline or saline on receptor densities in rat heart. Whilst the full agonist isoprenaline downregulated both β adrenoceptor subtypes, xamoterol had no effect on either. Xamoterol could have had an effect on receptor function in the absence of changes in receptor density by uncoupling receptors from adenylate cyclase. β adrenoceptors have a high affinity for agonists when part of a ternary complex of agonist, receptor and G-protein. GTP and its analogues dissociate the α subunit of the G-protein and return the receptor to a low affinity state. Thus receptors exist in 2 agonist affinity states depending on their coupling to G-proteins. In Kowalski's rat model, the GTP analogue Gpp(NH)p produced a shift in receptor affinity for isoprenaline (to a lower affinity) in cardiac membranes from rats pretreated with xamoterol,

demonstrating that they were still coupled to G-proteins. However no direct assessment of adenylate cyclase activity was made in this study. Furthermore there was no information on the subtype coupling to adenylate cyclase.

There is no direct data on the modulation of receptor regulation by xamoterol in human cardiac tissue. Since there are considerable species differences in the intrinsic activity of xamoterol, direct extrapolation from animal data may not be representative of human data. The data is of direct clinical relevance. Xamoterol should not desensitise cardiac β adrenoceptors if tolerance to its agonist properties is not to occur. An effect on β adrenoceptors similar to that of β adrenoceptor antagonists could suggest that xamoterol may also produce the beneficial effects of these drugs in selected patients with heart failure, perhaps with the advantage of exhibiting less negative inotropism at rest. It was the primary aim of the research presented in this thesis to investigate the effect of xamoterol on the regulation of human cardiac β adrenoceptors *in vivo*.

1.7 Outline of Research

Cardiac tissue had to be obtained from a group of patients in whom there was an ethically acceptable indication for xamoterol therapy. Patients with ischaemic heart disease and stable angina pectoris undergoing coronary artery bypass surgery represent such a group of patients since xamoterol does have anti-anginal properties and it is possible to obtain a piece of right atrial appendage at the time of surgery. Furthermore, there could be potential benefits during the peri-operative period in using a partial agonist as

opposed to a full antagonist (less risk of myocardial suppression or significant brady-arrhythmias).

Unfortunately this model has some limitations. The majority of patients undergoing coronary artery bypass grafting are being treated with β blockers. Ethical constraints prevent the inclusion of a group of patients taking neither β blockers or xamoterol as this would entail withdrawing β blockade entirely in patients with severe ischaemic heart disease shortly before surgery. Accordingly the effect of xamoterol therapy had to be compared with that of a β adrenoceptor antagonist. Atenolol was chosen as there is already published data on the effect of this drug on β adrenoceptor density and coupling in human right atrium. Another potential problem was the fact that coupling of β_1 adrenoceptors to adenylate cyclase is hard to demonstrate in human cardiac tissue. Thus the human model was unlikely to produce a complete picture of the pharmacological effects of chronic *in vivo* therapy with xamoterol on cardiac β adrenoceptors. For these reasons an animal model was developed in which coupling of both receptor subtypes could be demonstrated and the effect of xamoterol therapy could be compared both with the effect of no treatment and also with treatment with a full agonist.

The objectives of the research were therefore as follows.

1 To develop an assay to measure β adrenoceptor subtype density in both human atrial and rat ventricular tissue.

2. To develop an assay to measure β adrenoceptor subtype coupling to adenylate cyclase.

3 To apply these assays to investigate the effects of chronic xamoterol therapy on the subtype regulation of human and rat cardiac β adrenoceptors density and coupling to adenylate cyclase.

4 To determine whether xamoterol therapy conferred any benefit on the peri-operative course of patients undergoing coronary artery bypass surgery when compared to atenolol therapy.

Chapter 2

Radioligand Binding Assay

2.1 Introduction

The basic principle of radioligand binding is relatively simple. A radioactively labelled ligand (hormone or drug, agonist or antagonist, that has a high affinity and specificity for the receptor of interest) is incubated with a tissue preparation containing receptors. At equilibrium, unbound radioligand is removed and receptor bound ligand determined.

The radioligand should have a high affinity for the receptor under investigation so that low concentrations may be used, minimising non-specific binding (to sites other than the receptor) which is dependent on ligand concentration. Since receptor numbers are small, the isotope used to label the ligand should be of a high specific activity. For this reason, ^{125}iodine with a specific activity of 2175 $Ci.mmol^{-1}$ is commonly used. The radioligand should react to receptors with a uniform affinity to avoid complex binding phenomena that may be difficult to interpret. Receptor antagonists are commonly used as radioligands as there may be more than one receptor affinity state for agonists.

[^{125}I]iodopindolol meets the above criteria and was used for this study. This radioligand has been extensively used to label β adrenoceptors in various tissues both in this laboratory and elsewhere. It is a β adrenoceptor antagonist with a high affinity for both β adrenoceptor subtypes with minimal selectivity for the β_2 subtype (Barovsky and Brooker, 1980; Neve *et al*, 1986).

A non-selective ligand will label both β adrenoceptor subtypes. There are various possible approaches available to quantify receptor subtypes. Occasionally a subtype-specific ligand exists and can be used to label only

one subtype. [^{3}H]bisoprolol has been used in this way to identify β_1 adrenoceptors (Brodde *et al*, 1986). However since this technique only identifies β_1 adrenoceptors, further experiments are required to determine total receptor densities in order to quantify the other subtype.

A more commonly used method is to label both subtypes with a non-selective radioligand which is displaced by increasing concentrations of an unlabelled subtype-selective ligand. At low concentrations, the radioligand will be displaced from the subtype for which the unlabelled ligand has a high affinity whereas at higher concentrations the radioligand will be displaced from both subtypes. Using computer assisted analysis of such displacement curves it is possible to define the ratio of receptor subtypes. The most commonly used subtype-selective displacing ligands in the study of β adrenoceptor subtypes are the β_2 selective antagonist **ICI** 118,551 (Bilski *et al*, 1983) and the β_1 selective antagonist CGP 20712A (Dooley *et al*, 1986). Of the 2 antagonists, CGP 20712A is the more subtype-selective with an affinity for the β_1 subtype that is between 1,000 and 10,000 greater than that for the β_2 subtype. This technique requires numerous data points and still requires a further experiment to determine the total receptor densities. This is a potential disadvantage if the quantity of tissue is limited.

An alternative approach is to use an appropriate concentration of an unlabelled subtype-selective ligand to occupy one receptor subtype leaving the other subtype alone available to the non-selective radioligand. Since assays in the presence and absence of such a subtype-selective agent can be performed in the same experiments, this technique is more economical both in terms of tissue and time. β adrenoceptor subtype ratios have been determined previously using a concentration of CGP 20712A expected to occupy nearly all β_1 adrenoceptors and virtually none of the β_2 adrenoceptors

(Chester *et al*, 1992; Kaumann and Lemoine, 1987a; Kowalski *et al*, 1990). It was anticipated that tissue availability in the human study would be limited and thus this approach was investigated.

This chapter contains details of the methods used to prepare sarcolemnal membranes from cardiac tissue and for the iodination of pindolol. This is followed by data to validate the binding of [^{125}I]iodopindolol to β adrenoceptors in cardiac tissue and experiments to determine an appropriate concentration of CGP 20712A to selectively inhibit [^{125}I]iodopindolol binding to β_1 adrenoceptors.

2.2 Methods

2.2.1 Membrane preparation

Human right atria

Right atrial appendages were excised at open heart surgery immediately prior to the initiation of cardiopulmonary bypass. The biopsy was immediately placed in ice cold normal saline and transported to the laboratory. The specimen was washed of blood, dissected of fat and homogenised in ice cold buffer (1 mM $KHCO_3$) with an Ultra Turrax homogeniser for 20 seconds at full speed and twice for 20 seconds at half speed. The homogenate was passed through 4 layers of gauze and centrifuged for 20 minutes at 50,000g at 4°C. The pellet was resuspended in ice cold buffer to wash the membranes and centrifuged again before final resuspenslon in ice cold buffer. The resulting crude membrane preparation was divided into aliquots and frozen and stored at minus 70°C. It has previously been shown that membranes could be

stored in such conditions for prolonged periods without significant loss of binding sites (Dickinson, 1982; Hershberger *et al*, 1991).

Rat Hearts

Rats were sacrificed either by cervical dislocation or exsanguination under general anaesthesia with Halothane and the heart removed and immediately placed in ice cold normal saline. The great vessels, atria and right ventricles were excised and the left ventricle bisected and washed of blood. The left ventricle was then homogenised in ice cold buffer (1 mM $KHCO_3$) and prepared as for the human right atria above.

2.2.2 Iodination of (-)pindolol

(-)Pindolol was iodinated according to the method of Barovsky and Brooker (1980). 5 µl of 10 mM (-)pindolol in ethyl alcohol was placed in an eppendorf microcentrifuge tube and evaporated to dryness under a stream of nitrogen. 1-2 mCi of $Na^{125}I$ (Amersham International) was added followed by 40 µl of 0.3 M potassium phosphate buffer pH 7.5 and 5 µl of aqueous chloramine-T solution (0.17 mg/ml). The tube was vortexed briefly and the iodination allowed to continue for three minutes at room temperature. The reaction was stopped with 500 µl of sodium metabisulphite (1 mg/ml in 1M acetic acid). The mixture was adjusted to pH 10 with 2N sodium hydroxide. Pindolol and its iodinated derivative were extracted five times with 300 µl of ethyl acetate. Labelled and unlabelled pindolol were then separated by paper chromatography. The extract was reduced in volume to approximately 300 µl under a stream of nitrogen and spotted onto a strip of Whatmann 3MM paper. The chromatogram was developed in ammonium formate (pH 8.5): methanol,

10:1 (v:v) at room temperature for approximately 3 hours (until the solvent front had run approximately 30 cm). The chromatography paper was cut into 1 cm strips which were then eluted in 5 mls of ethyl acetate containing 2% diethylamine (v:v). 5 µl aliquots of the eluent from each strip were counted to locate the peak of [^{125}I]iodopindolol, usually contained in 2 or 3 fractions. These were then pooled and stored in glass vials at minus 20°C following the addition of 1 µl of 1% phenol in ethyl acetate per ml.

Following each iodination the purity of [^{125}I]iodopindolol was tested by performing a saturation binding assay against a standard preparation of rat lung membranes.

2.2.3 [^{125}I]iodopindolol binding assays

100 µl of myocardial membranes diluted if necessary with 1 mM $KHCO_3$ to contain approximately 50 µg protein in rat experiments and 30 µg protein in human experiments were added to [^{125}I]iodopindolol and appropriate concentrations of displacing agent or assay buffer to a final volume of 250 µl. For competition experiments [^{125}I]iodopindolol was added at a fixed concentration of approximately twice the affinity constant. For saturation experiments [^{125}I]iodopindolol was added at 6 different concentrations ranging from 6 to 250 pM. Incubations were performed in triplicate for competition experiments and duplicate for saturation experiments at room temperature for 60 minutes after which membranes were collected on Whatman GF/B filters using a Brinkman multiport harvester and rapidly washed 4 times with 5 mls of cold assay buffer. Radioactivity retained on the filters was determined directly in a Packard Auto Gamma 500C counter at 72-74% efficiency.

Protein was estimated by the method of Lowry (Lowry *et al*, 1951) using bovine serum albumin as standards.

Competition curves were analysed by non-linear regression using the computer software package InPlot (GraphPad Software, San Diego) and an IBM compatible personal computer. Receptor densities were calculated by Scatchard transformation of saturation binding data (Scatchard, 1949).

2.3 Results

2.3.1 Specificity and saturability of [^{125}I]iodopindolol binding

Fig 2.1 shows the competition of (-)isoprenaline for binding of [^{125}I]iodopindolol (66 pM) to human right atrial membranes from a patient not taking beta blockers. The curve plateaus at approximately 14% of total binding. The binding which is not displaced by isoprenaline represents binding to sites other than β adrenoceptors, termed non-specific binding. This was defined in subsequent experiments as that binding remaining in the presence of 200 μM isoprenaline and was characteristically less than 10% of total binding at free concentrations of [^{125}I]iodopindolol equal to its Kd..

Receptor numbers vary with amount of membrane added in experiments. Therefore specific binding to receptors should be directly proportional to protein concentration, whereas non-specific binding tends to be proportional to the concentration of radioligand. Fig 2.2 confirms that specific binding of [^{125}I]iodopindolol to human right atrial membranes was linear over protein concentrations from 10 to 50 μg.

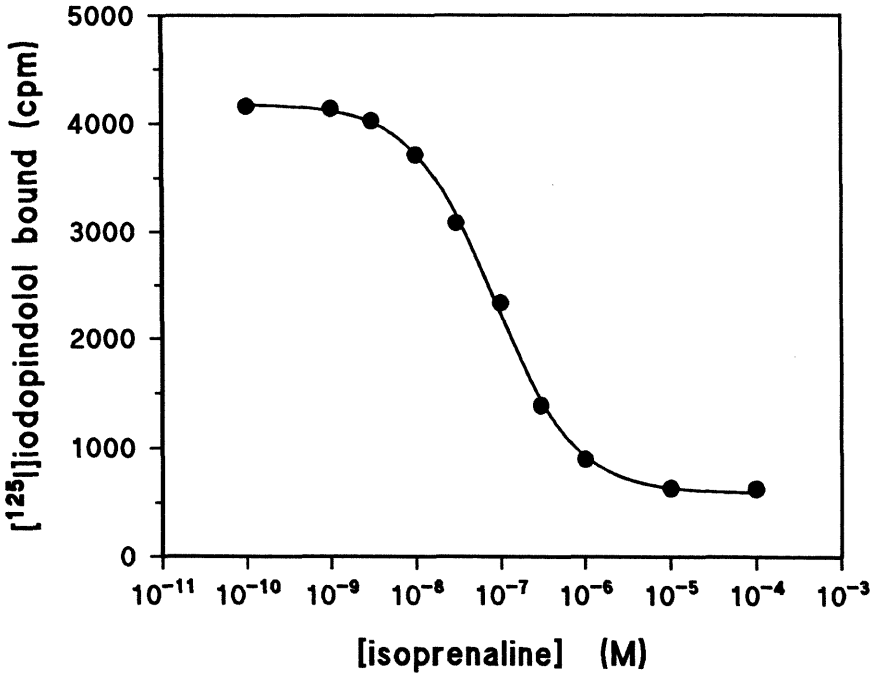


Fig 2.1 Displacement of [^{125}I]iodopindolol (66 pM) from human right atrial membranes by increasing concentrations of the non-selective β adrenoceptor agonist isoprenaline. IC_{50} = 83 nM.

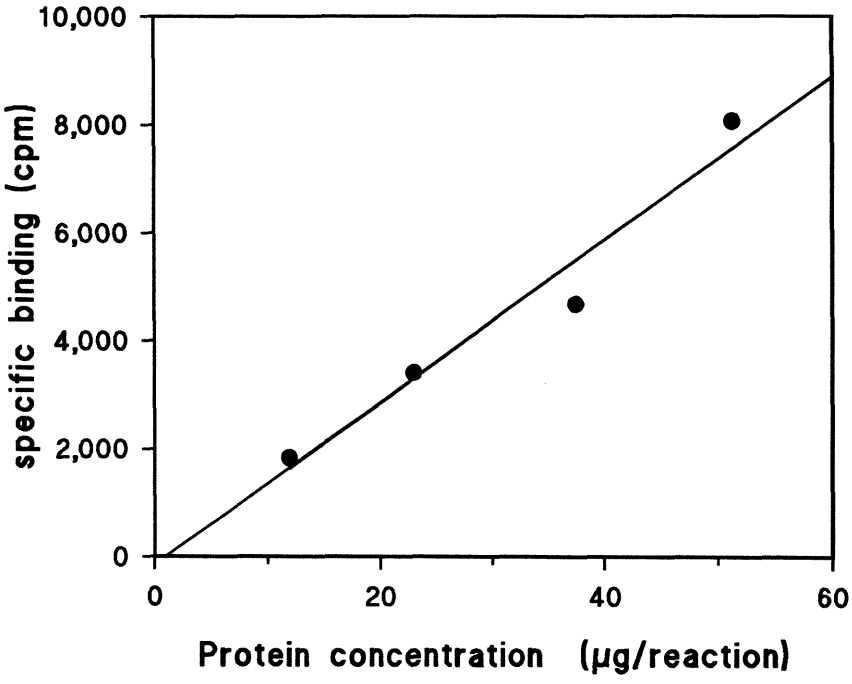


Fig 2.2 Effect of protein concentration, expressed as µg of protein per reaction, on the specific binding of [^{125}I]iodopindolol, expressed as counts per minute, to human right atrial membranes. r = 0.98.

If the radioligand is binding to β adrenoceptors, it should be displaced by the stereoisomers of a drug with an affinity that would be expected from the stereoisomers' biological activity at β adrenoceptors. Competition experiments showed that the stereoisomers of propanolol displaced [^{125}I]iodopindolol binding with different affinities as expected from their biological activities with the active (-)-isomer having a higher affinity than the (+)-isomer (fig 2.3).

The number of receptors in a given tissue are finite and thus binding of radioligands should be saturable. Fig 2.4 shows typical curves of [^{125}I]iodopindolol binding to membranes prepared from human right atrial appendage. The data points describe a rectangular hyperbole as would be expected from the Law of Mass Action. Fig 2.5 shows a Scatchard plot of the same data. The plot is linear, which confirms that binding is to one class of receptors only and therefore that the ligand is not significantly selective for one of the receptor subtypes.

Thus the binding of [^{125}I]iodopindolol to β adrenoceptors in human right atrial membranes is saturable, of high specificity, linear with protein concentration and stereo-selective.

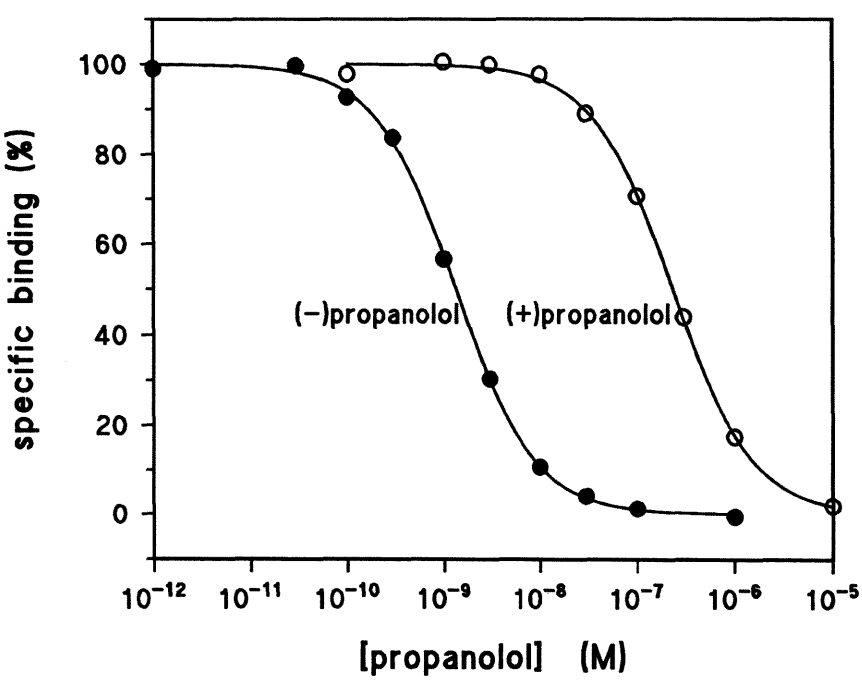


Fig 2.3 Competition of the 2 stereoisomers of propanolol for the binding of [^{125}I]iodopindolol (79 pM) to rat heart membranes. IC_{50} for (-)propanolol = 1.34 nM (●), IC_{50} for (+)propanolol = 234 nM (O)

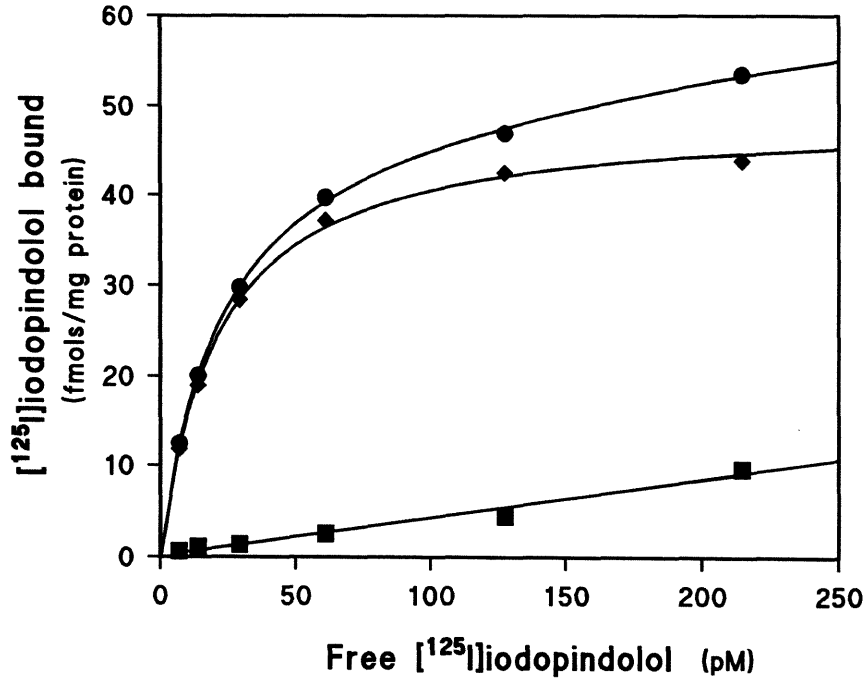


Fig 2.4 Binding of [^{125}I]iodopindolol to human right atrial membranes. ● total binding; ■ non specific binding; ◆ specific binding. Data shown are of a typical experiment performed in duplicate.

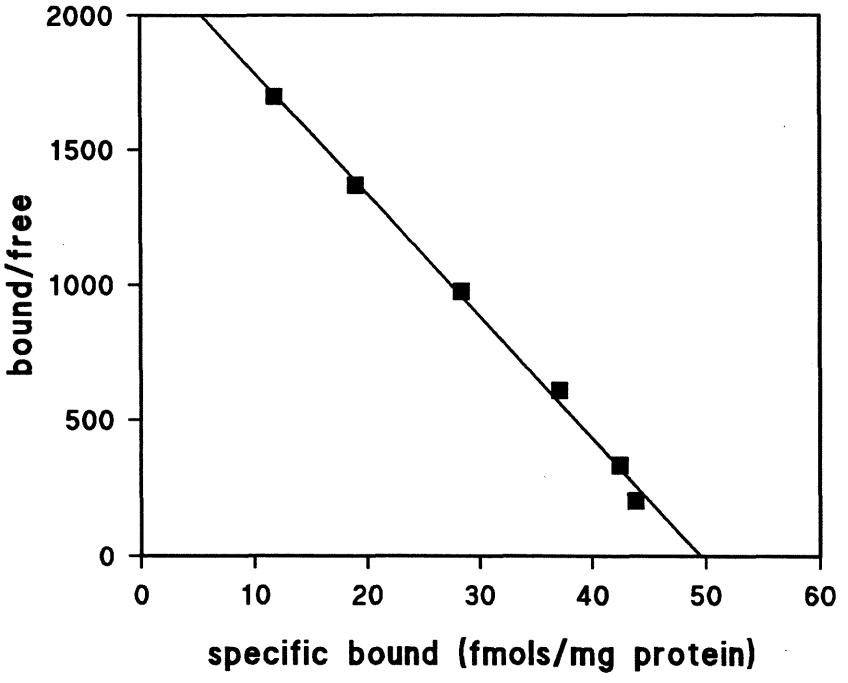


Fig 2.5 Scatchard plot of data from fig 2.4. B_{max} = 49.55 fmol/mg protein, K_d = 22.07 pM.

2.3.2 Quantification of β adrenoceptor subtypes

Competition curves of CGP 20712A for [^{125}I]iodopindolol binding were best fit using a 2 site model in both human right atria (fig 2.6) and rat left ventricle (fig 2.7) indicating the presence of 2 receptor subtypes in both preparations. The affinity of CGP 20712A for the 2 sites may be determined by the Cheng and Prussof equation (Cheng and Prussoff, 1973) as follows

$$K_i = \frac{IC_{50}}{1+\frac{L}{K_d}}$$

where K_i is the affinity constant for CGP 20712A, IC_{50} the concentration of CGP 20712A required to inhibit 50% of the binding to a receptor subtype, L the concentration of [^{125}I]iodopindolol and K_d the affinity of [^{125}I]iodopindolol for the receptor subtype. Substituting a K_d of 30 pM for [^{125}I]iodopindolol binding to β_1 adrenoceptors (equivalent to that characteristically found in saturation experiments in cardiac tissue with predominantly β_1 receptors) and 15 pM for β_2 adrenoceptors (assumes a 2 fold selectivity for the β_2 subtype), this would give a K_i for β_1 adrenoceptors of 1.6 nM in human right atria and 1.2 nM in rat ventricle and for β_2 adrenoceptors of 1.2 μM and 1.3 μM respectively. These values were consistent with previously published reports of the affinity of CGP 20712A for β_1 and β_2 receptors (Kaumann and Lemoine, 1987b).

According to the Law of Mass Action, receptor occupancy (R) is related to ligand concentration (L) and affinity of ligand for receptor (K_d) by the formula

$$R = \frac{L}{L + K_d}$$

From the affinity constants determined above it is possible to estimate the subtype occupancy at varying concentrations of CGP 20712A (table 2.1). It was decided to investigate the use of 100 nM since this was the lowest concentration to effectively block β_1 adrenoceptors without a major effect on β_2 adrenoceptors.

Table 2.1 β adrenoceptor subtype occupancy by various concentrations of CGP 20712A. Assumes K_d β_1 of 1.4 mM and K_d β_2 of 1.25 μM

Concentration of CGP 20712A (nM)	β_1 occupancy (%)	β_2 occupancy (%)
10	88	1
30	95	2
100	99	7
300	99	19
1000	100	44

To test if this was an appropriate concentration to use in saturation experiments to determine the subtype proportions, the results from this technique were compared with those obtained using full competition curves in 5 rat hearts (fig 2.8 and 2.9). There was no significant difference in the subtype proportion using the 2 methods. β_1 adrenoceptors represented 72.1 ± 1.5 % (mean ± sd) of total receptors as estimated by the saturation binding experiments and 71.0 ± 2.7% by the full displacement curves.

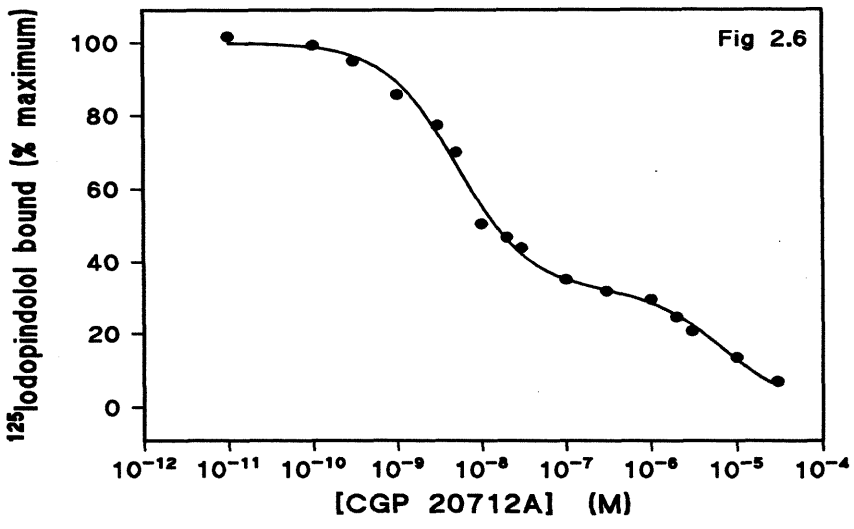


Fig 2.6 Competition of CGP 20712A for [^{125}I]iodopindolol binding. Human right atrium, mean of 2 experiments performed in triplicate. IC_{50} high affinity = 5.19 nM, low affinity = 6.67 μM. Added [^{125}I]iodopindolol = 70 pM. K_i β_1 = 1.60 nM, K_i β_2 = 1.19 μM. β_1 adrenoceptors represent 67.6% of total receptors.

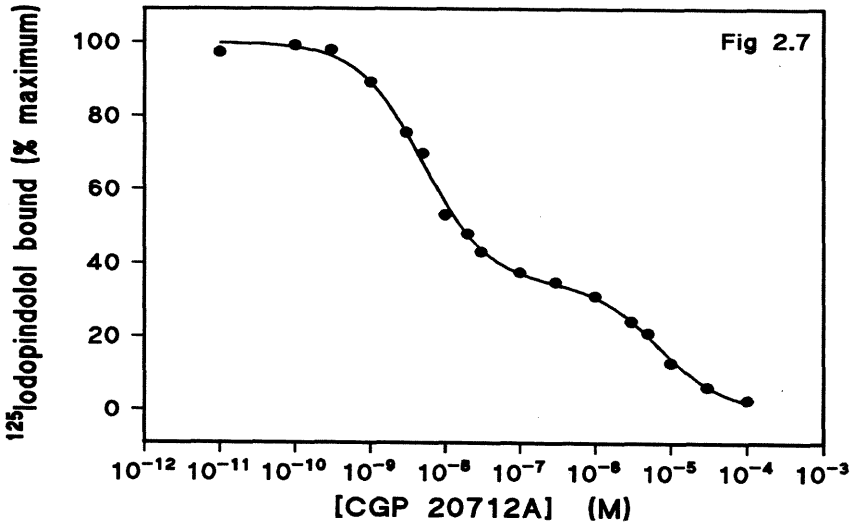


Fig 2.7 Competition of CGP 20712A for [^{125}I]iodopindolol binding. Rat left ventricle, mean of 3 experiments performed in triplicate. IC_{50} high affinity = 3.91 nM, low affinity = 7.61 μM. Added [^{125}I]iodopindolol = 68 pM. K_i β_1 = 1.20 nM. K_i β_2 = 1.29 μM. β_1 adrenoceptors represent 65.4% of total receptors.

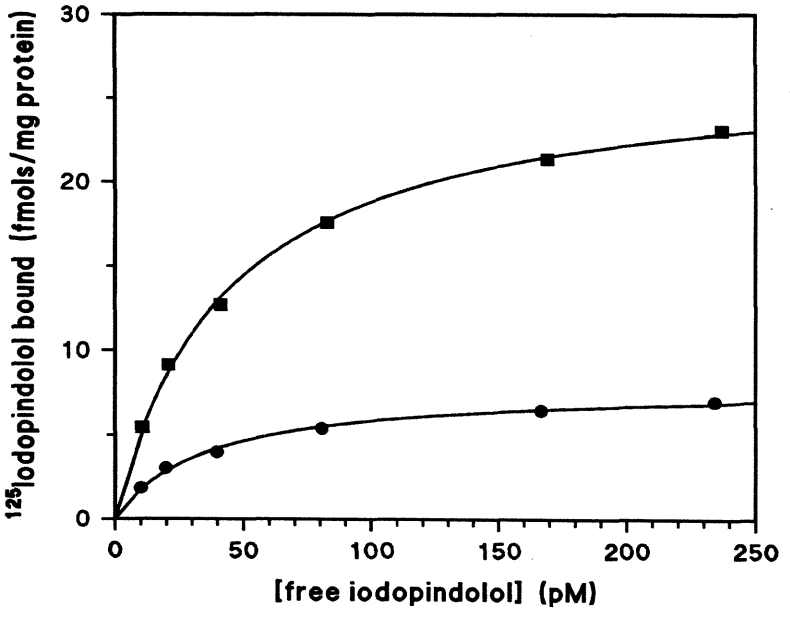


Fig 2.8 Typical saturation binding experiment using rat left ventricular membranes. Upper curve (■) shows specific binding of [^{125}I]iodopindolol in the absence of CGP 20712A representing binding to both β_1 and β_2 adrenoceptors. Lower curve (●) shows specific binding of [^{125}I]iodopindolol in the presence of 100 nM CGP 20712A representing binding to β_2 adrenoceptors only. B_{max} (fmols/mg protein) : total = 26.31; β_2 = 7.74 , β_1 = 18.57. β_1 adrenoceptors represent 70.6 % of total.

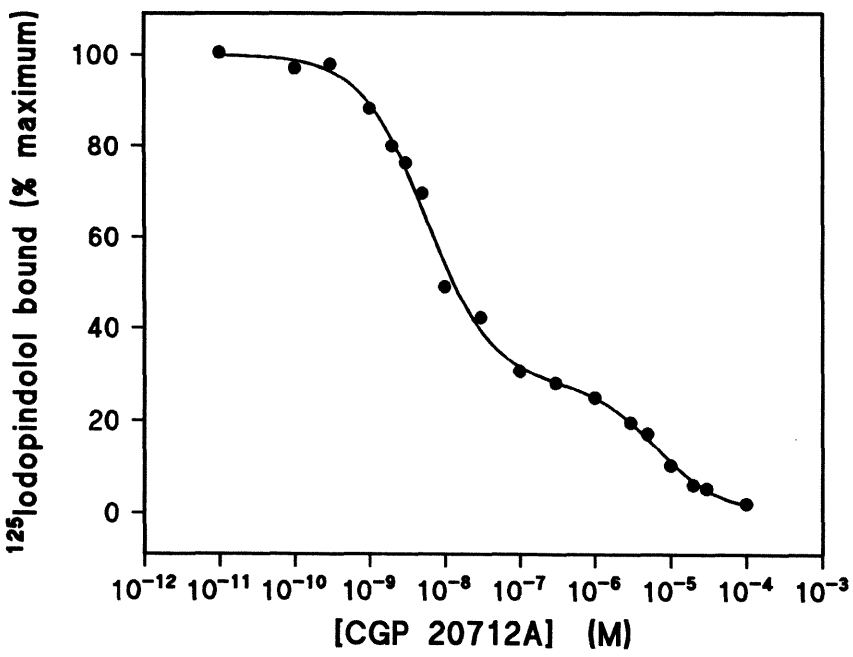


Fig 2.9 Typical experiment of the competition of CGP 20712A for the binding of [^{125}I]iodopindolol to rat left ventricular membranes. Data obtained using membranes from the same animal as in figure 2.8. β_1 adrenoceptors represent 71.8 % of total.

2.4 Conclusions

The data presented in this chapter confirm that [^{125}I]iodopindolol as produced in this laboratory selectively binds to cardiac β adrenoceptors with a high specificity. Both β adrenoceptor subtypes are present in homogenates of human right atrium and rat left ventricle. CGP 20712A at a concentration of 100 nM effectively inhibits binding to β_1 adrenoceptors and may be used in saturation experiments to determine receptor subtype proportions in situations where tissue availability is limited.

Chapter 3

Adenylate Cyclase Assay

3.1 Introduction

3.1.1 Coupling of β adrenoceptors to adenylate cyclase

There is good evidence that cyclic AMP (cAMP) is involved in the inotropic response to β adrenoceptor stimulation in man as in other mammals. Ikenozo *et al* (1987) showed that in human heart intracellular cAMP levels rise in response to β adrenoceptor stimulation and that this rise precedes the physiological response. Furthermore, they demonstrated that inhibition of cAMP breakdown with phosphodiesterase inhibitors enhances the effects of β adrenoceptor agonists on both cAMP production and force of contraction. Dibutyryl cAMP, an active analogue of cAMP that can cross cell membranes, mimics the effects of β adrenoceptor agonists suggesting a common mechanism (Hall *et al*, 1990).

cAMP is produced from ATP by the membrane bound enzyme adenylate cyclase. The ability to isolate the β adrenoceptor from this enzyme (Haga *et al*, 1977; Limbird and Lefkowitz, 1977) and subsequent reconstitution experiments revealed that a third component was required for signal transduction (Pfeuffer, 1977; Ross and Gilman, 1977) This was subsequently shown to be a guanine-nucleotide binding protein (G-protein) (Northup *et al*, 1980; Sternweis *et al*, 1981) of which a large family of related proteins have now been identified. They are heterotrimeric proteins possessing α, β and γ subunits. They are activated by the binding of GTP and remain active until bound GTP is hydrolysed to GDP by intrinsic GTPase activity. A stimulatory protein, G_s, and an inhibitory protein, G_i, are involved in the transduction of β adrenoceptor signalling. They differ in their α subunit but share similar β and γ subunits.

A ternary model (Gilman, 1987) is thought to explain the signal transduction process (fig 3.1). GDP is bound to the α subunit of G_s ($G_{s\alpha}$) in its inactive state. Upon interaction with a specific receptor activated by an agonist, GDP is released and GTP bound. This causes dissociation of the α subunit from the β and γ subunits. The activated α subunit stimulates the effector molecule adenylate cyclase to produce cAMP from ATP. The intrinsic GTPase activity of the α subunit hydrolyses bound GTP to produce GDP, rendering it unable to stimulate adenylate cyclase further and dissociating it from the enzyme. The α subunit then recombines with the free $\beta\gamma$ subunits to form the inactive trimer to be recycled. G_s can exchange $\beta\gamma$ subunits with G_i. One possible mechanism for inhibition of adenylate cyclase by G_i is that free $\beta\gamma$ units released following the activation of G_i combine with free $G_{s\alpha}$ subunits rendering them inactive.

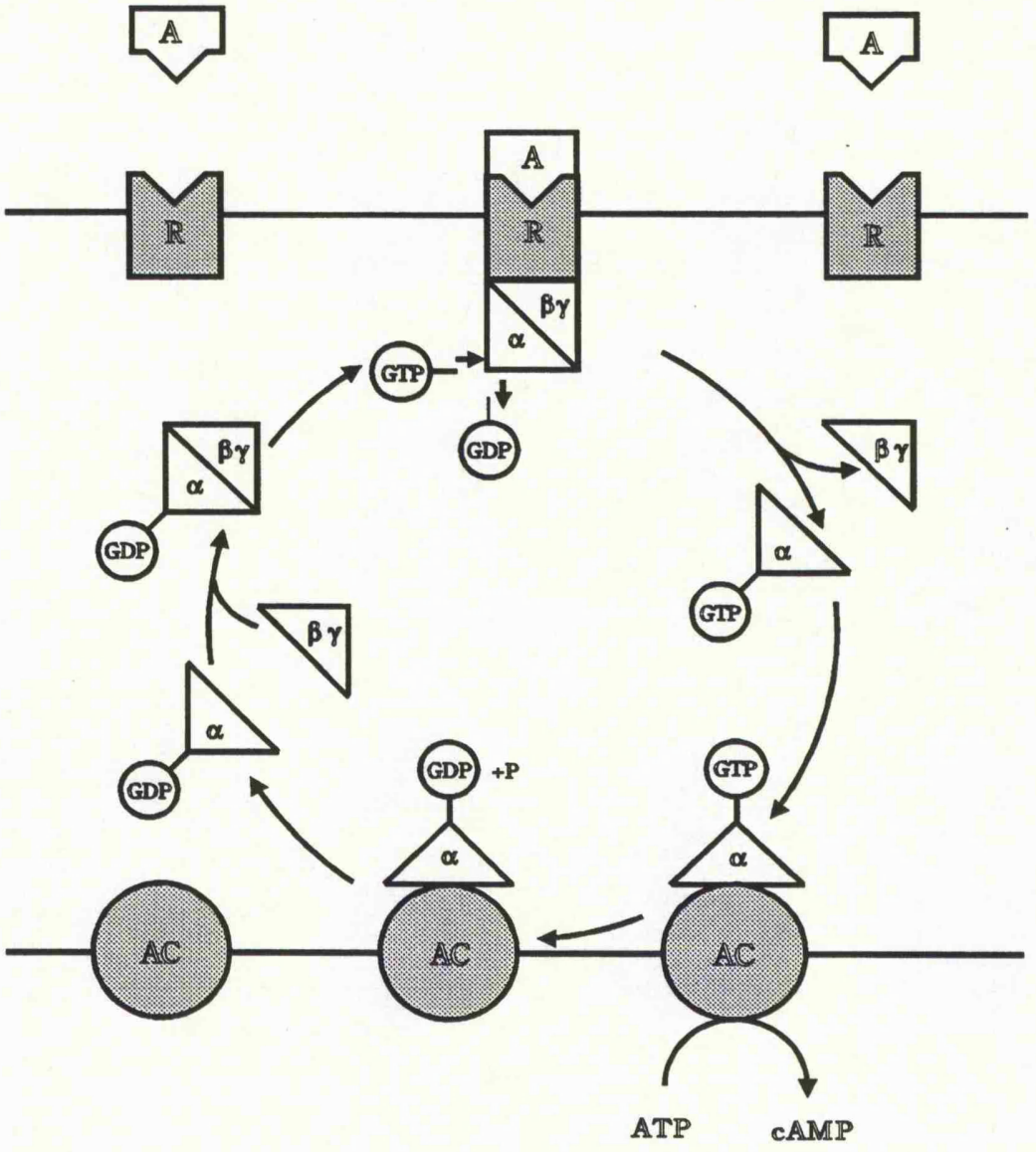


Fig 3.1 Schematic representation of the coupling of β adrenoceptors to adenylate cyclase. "A" represents a receptor agonist outside the cell membrane, "R" the receptor on the extracellular surface of the membrane and "AC" the enzyme adenylate cyclase on the inner surface of the cell membrane. The α subunit of G_s is represented by a triangle labelled α and the β and γ subunits are represented by a triangle labelled βγ.

It is possible to probe the various components of the pathway in vitro. GTP or its analogues may stimulate the G-proteins even in the absence of an activated receptor. The response of adenylate cyclase to stimulation by GTP will give an indication of overall G-protein function. Although it will not differentiate between G_i or G_s activity, in functional terms it is the balance of G_i and G_s activity that is relevant. The non-hydrolysable analogue Gpp(NH)p is sometimes used to stimulate G-proteins but this has the disadvantage of irreversibly activating adenylate cyclase and in time would maximally activate the enzyme. In the following experiments G-protein function will be assessed using GTP. An alternative to GTP would be to use the fluoride ion, but this selectively activates G_s.

In the presence of GTP, receptor mediated stimulation of the pathway can be determined using receptor agonists. Maximal receptor mediated stimulation will be produced by using a maximally effective concentration of a non-selective β adrenoceptor agonist such as isoprenaline. In some tissues a maximal response to a full agonist may occur at less than full receptor occupancy in which case there are said to be "spare" receptors. For a partial agonist a maximal effect will only occur at full receptor occupancy.

No entirely satisfactory method has been found to assess the catalytic moiety of adenylate cyclase itself. The diterpine forskolin can directly activate adenylate cyclase in the absence of G-proteins. However the presence of Gs enhances this effect (Seamon, 1985) and thus the response to forskolin is not entirely specific for the adenylate cyclase molecule.

It is an unexplained anomaly of human myocardium that whilst β_1 adrenoceptors outnumber β_2 adrenoceptors and mediate the greater part of the inotropic response to agonist stimulation (Lemoine *et al*, 1988), adenylate

cyclase activation is predominantly mediated by β_2 adrenoceptors. Waelbroeck *et al* (1983) were unable to demonstrate any coupling of β_1 adrenoceptors to adenylate cyclase in membranes prepared from human atria using a variety of agonists and selective antagonists. Bristow *et al* (1989) could only demonstrate stimulation of adenylate cyclase with the β_1 adrenoceptor partial agonists prenalterol and denopamine in human ventricular membranes by amplifying the signal using forskolin and Gpp(NH)p. β_2 adrenoceptor coupling was easily demonstrated without these agents. Gille *et al* (1985) found that selective blockade of the β_2 subtype with ICI 118,551 did not inhibit adenylate cyclase stimulation by low concentrations of the relatively β_1 selective catecholamine noradrenaline and suggested that the ICI 118,551 resistant fraction was due to stimulation of β_1 adrenoceptors. Even in these experiments, β_2 adrenoceptor coupling was greater than β_1 adrenoceptor coupling.

There are several possible explanations for the discrepancy between β_1 adrenoceptor mediated stimulation of adenylate cyclase and mediation of positive inotropic responses.

Firstly, β adrenoceptor mediated inotropic responses may not be exclusively mediated by cAMP generation. There is evidence that G_s can directly activate calcium channels (Yatani *et al*, 1987) and Lemoine and Kaumann (1991) have recently suggested that there may be a component of β_2 adrenoceptor mediated positive inotropic effect that is unrelated to cAMP in cats. However Hall *et al* (1990) found that stimulation of either β_1 or β_2 adrenoceptors in human right atrial strips shortened the time to peak tension, a characteristic feature of cAMP mediated inotropic effects.

Secondly, β_1 adrenoceptors may be coupled to adenylate cyclase in vivo but may become uncoupled in the preparation of the membranes. However similar membrane preparations from other species (including the rat as demonstrated below) have easily demonstrable coupling of β_1 adrenoceptors.

Thirdly, such a small amount of cAMP may be required to produce an inotropic response that it is below the level of detection. However unlike feline myocardium there are few "spare" receptors in human myocardium in which there is a close relationship between receptor occupancy, cAMP production and inotropic response (Brown *et al*, 1992; Kaumann and Lemoine, 1987)

Fourthly, in homogenates, myocytes may be contaminated with non-muscle cells rich in β_2 adrenoceptors. Using angiotensin converting enzyme as a marker for endothelial cell membranes, Tomlins *et al* (1986) demonstrated that up to 42% of membranes in an homogenate of sheep heart may be from contamination with endothelial cells. If these non-myocytes are strongly coupled to adenylate cyclase, the cAMP produced by them may mask that produced by myocytes.

Finally, there may be only a small subgroup of cells that produce cAMP via β_1 adrenoceptor stimulation with subsequent propagation of inotropic effects to surrounding cells by non cAMP mediated mechanisms.

Regardless of the cause for the discrepancy, it does place some constraints on the interpretation of experiments in human myocardium. Since coupling of β_1 adrenoceptors to adenylate cyclase is difficult to demonstrate, drug or disease induced changes in this will be difficult to follow. Furthermore since

coupling of β_1 adrenoceptors to adenylate cyclase correlates poorly with inotropic responses in human heart tissue, it would be difficult to interpret the functional significance of any observed changes in β_1 coupling. An alternative model was therefore required to study drug induced changes in the coupling of β_1 adrenoceptors to adenylate cyclase. In the rat heart both receptor subtypes coexist (Vago *et al*, 1984) and inotropic responses appear to be entirely mediated by the β_1 subtype (Juberg *et al*, 1985). It might therefore represent a useful model to look at the effects of drugs on β_1 adrenoceptor coupling to adenylate cyclase.

This chapter describes the methods used in the adenylate cyclase assay followed by a description of experiments designed to investigate the characteristics of β adrenoceptor subtype coupling to adenylate cyclase in membrane preparations from human right atrium and rat left ventricle.

3.1.2 Principles of adenylate cyclase assays

The basic principle involved in measuring adenylate cyclase activity is straightforward. Excess ATP is incubated with a membrane preparation containing the enzyme and the amount of cAMP produced assayed. Since some of the phosphodiesterase isoenzymes are also membrane bound, a phosphodiesterase inhibitor such as IBMX is included in the incubation buffer to prevent the breakdown of cAMP. Membranes are also a rich source of ATPase, which in long incubations will significantly reduce the concentration of the substrate ATP. For this reason a regenerating system is included comprising creatine phosphate as a source of phosphate and creatine

phosphokinase to catalyse the conversion of ADP to ATP using the phosphate from creatine phosphate.

The techniques used to assay cAMP fall broadly into 2 categories. In the first, [^{32}P]-labelled ATP is used as a substrate and production of [^{32}P]cAMP measured. Since ATP is added in excess, labelled ATP greatly exceeds the concentration of [^{32}P]cAMP and a highly selective method for separating the 2 components is necessary. Salomon *et al* (1974) have described such a technique using chromatography on both Dowex and aluminium oxide columns. A tracer of [^{3}H]cAMP is usually added to estimate the recovery of cAMP in the separation process.

The alternative method to assay cAMP production is to use unlabelled ATP in the incubation and directly measure the concentration of cAMP produced. This method was chosen because the laboratory was not equipped for experiments with [^{32}P], whereas a competitive protein binding assay for cAMP, as described by Brown *et al* (1971), had previously been used in the laboratory. This uses an extract produced from bovine adrenals which contains a protein that avidly and selectively binds cAMP (probably protein kinase A). A fixed amount of this protein is labelled with a fixed concentration of [^{3}H]cAMP. The displacement of labelled cAMP from the protein by the cold cAMP in the sample is compared to the displacement produced by a series of standard concentrations of cold cAMP. The assay is sensitive but has a limited range. Varying amounts of cellular membranes have to be added in the incubation (depending on the adenylate cyclase activity) in order that the cAMP produced should be in the range of the assay. It is therefore important that the assay should be linear with added membrane protein. In addition other components of the incubation medium may interfere with the binding of

cAMP to the bovine binding protein and blanks of this medium may need to be added to the cold standards to allow for this.

3.2 Method

3.2.1 Preparation of bovine adrenal binding protein

Fresh bovine adrenals were obtained from a local abattoir. The medulla and capsule were removed and the cortices homogenised with an ultraturrax homogeniser in 1.5 volumes of Littlefields medium (sucrose 250 mM, Tris-HCl 50 mM, KCl 25 mM, $MgCl_2$ 5 mM, 2-mercaptoethanol 6 mM, theophylline hydrate 8 mM, adjusted to pH 7.4 with 1N HCl). The homogenate was centrifuged at 2,000g for 5 minutes at 4°C, the cell debris discarded and the supernatant re-centrifuged twice at 50,000g for a total of 40 minutes. The resulting supernatant was divided into 0.5 ml aliquots and stored at -20°C until required. Specific binding of [^{3}H]cAMP was linear with protein dilution (data not shown). A dilution that bound 30-40% of the total added [^{3}H]cAMP was used in the cAMP assays described above.

3.2.2 Adenylate cyclase assay

Adenylate cyclase activity was determined in triplicate at 37°C. Cardiac membranes (approximately 40 µg protein in rat and 15 µg in human experiments) were incubated in a total volume of 500 µl with final concentrations of 50 mM Tris pH 7.4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, 1 mM IBMX, 1 mM ascorbic acid and 1 mM ATP. Experiments were performed in the presence of 0.1 mM GTP unless otherwise stated. The concentrations of Mg^{++} and GTP were chosen from data produced by Golf *et al* (1984). All reagents with the exception of ATP were equilibrated for 5 minutes at 37°C before the reaction was initiated by the addition of ATP. The reaction was terminated after 15 minutes incubation by plunging the tubes into boiling water for 3

minutes. The samples were centrifuged (2,000g for 20 minutes) to spin down denatured protein and 50 µl aliquots were stored at minus 20°C for assay of cAMP.

cAMP was determined using the protein binding assay described above (Brown *et al*, 1971). 50 µl samples were incubated at 4°C with approximately 24,000 cpm [^{3}H]cAMP and 100 µl of bovine adrenal extract (see above) in a final volume of 400 µl of buffer (Tris 50 mM, EDTA 4 mM, pH 7.4). After 90 minutes the reaction was terminated by adding 250 µl of a charcoal suspension (0.25 gm charcoal, 1 ml of 10% bovine serum albumin and 50 mls of buffer) to adsorb unbound cAMP. After 10 minutes the samples were centrifuged at 2,000g for 20 minutes. 500 µl of supernatant were placed in 4 ml of scintillation fluid and counted for 5 minutes in a LKB 1216 Rackbeta liquid scintillation counter. To determine the cAMP content of the samples a standard curve was constructed using known concentrations of cold cAMP (0.125 to 8 pmoles cAMP in 50 µl buffer) in place of the samples. Preliminary experiments (fig 3.2) showed that the incubation medium used in the formation of cAMP did affect the binding of [^{3}H]cAMP to the binding protein and so for the standards a blank of 50 µl of incubation medium was added in the cAMP assay. cAMP concentrations in the samples were read from the standard curve constructed using a computer assisted iterative curve fitting program.

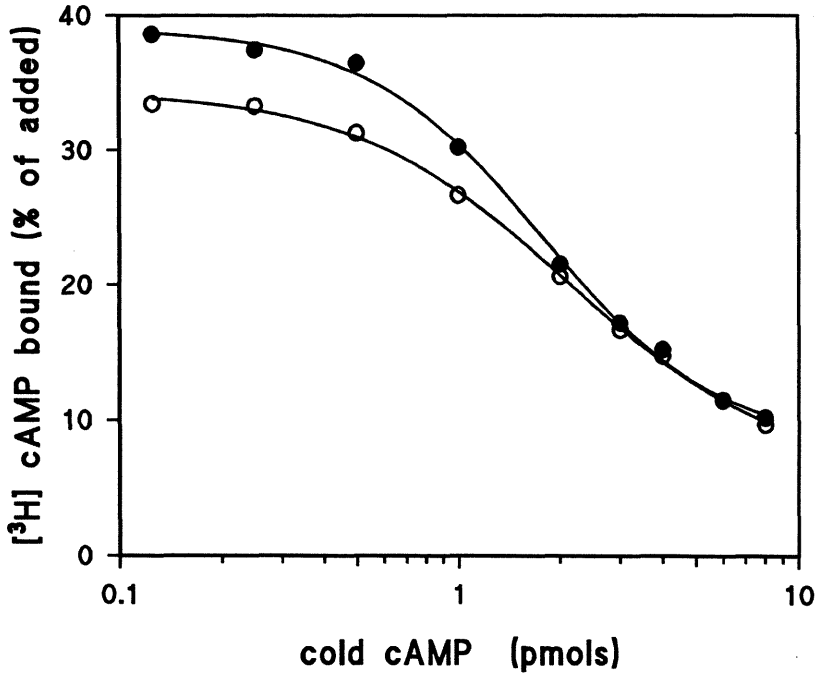


Fig 3.2 Standard curves for the displacement of [^{3}H]cAMP binding to bovine adrenal binding protein. ● = in the presence of buffer from membrane incubation to produce cAMP; ○ = in absence of incubation buffer

3.3 Results

3.3.1. Effects of protein concentration and time of incubation on cAMP production

cAMP production was linear with amount of added membrane protein (fig 3.3) and with the length of incubation of membranes (fig 3.4). In subsequent experiments incubations were performed for 15 minutes and adenylate cyclase activity expressed as pmols of cAMP per mg membrane protein per minute.

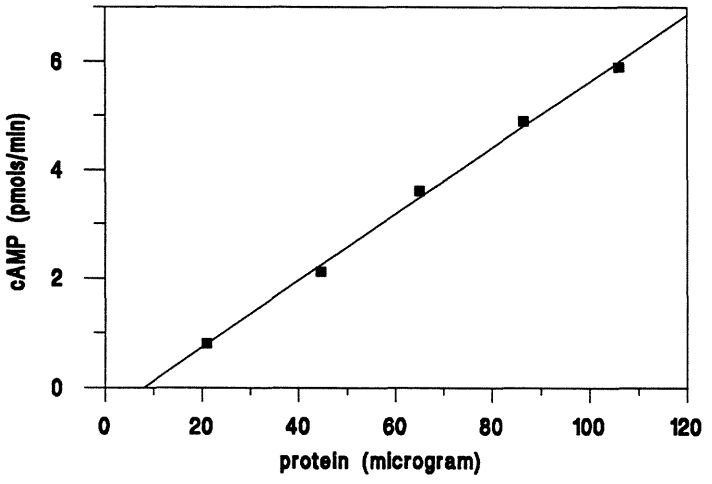


Fig 3.3 Effect of varying membrane protein on cAMP production from rat ventricular membranes stimulated with 10 μM isoprenaline. Representative experiment performed in triplicate.

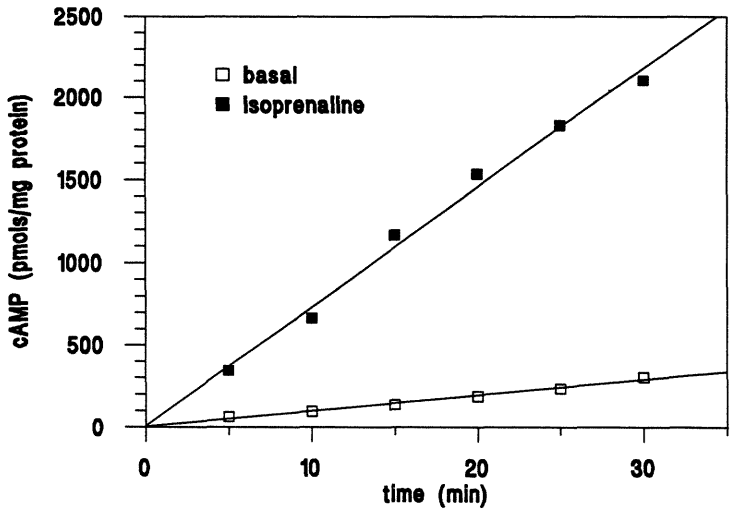


Fig 3.4 Time course for cAMP production from human right atrial membranes. Filled symbols represent basal activity and open symbols maximal stimulation with isoprenaline (10 μM). $r^2 = 0.99$ in both cases. Representative experiment performed in triplicate.

3.3.2 β adrenoceptor subtype coupling to adenylate cyclase in human right atrium

The original subclassification of β adrenoceptors was made on the relative potency of 3 catecholamines at producing various physiological effects (Lands *et al*, 1967). β_1 adrenoceptors were classified by a rank order of potency of isoprenaline > adrenaline ≥ noradrenaline whereas β_2 adrenoceptors were classified by the order of isoprenaline > adrenaline > noradrenaline. The rank order of potency of these 3 catecholamines at stimulating adenylate cyclase in membranes prepared from human right atria suggested that this was predominately mediated by the β_2 subtype (fig 3.5). This was confirmed by the use of subtype selective antagonists. Selective

inhibition of β_1 adrenoceptors by 100 nM CGP 20712A did not shift the dose response curve of stimulation of adenylate cyclase by the non-selective agonist isoprenaline. However a concentration of ICI 118,551 that selectively inhibits β_2 adrenoceptors (50 nM) shifted the dose response to the right (fig 3.6). The β_2 adrenoceptor partial agonist procaterol had a relatively high intrinsic activity, characteristically 75 to 80% that of isoprenaline demonstrating relatively strong coupling of β_2 adrenoceptors to adenylate cyclase in this preparation (fig 3.7).

An attempt was made to reproduce the experiments of Gille *et al* (1985) to dissect out the relative contributions of receptor subtypes to the stimulation of adenylate cyclase by noradrenaline. Whilst the β_2 adrenoceptor selective antagonist ICI 118,551 shifted the dose response of noradrenaline to the right, it proved impossible to reliably fit a biphasic curve to the points (fig 3.8).

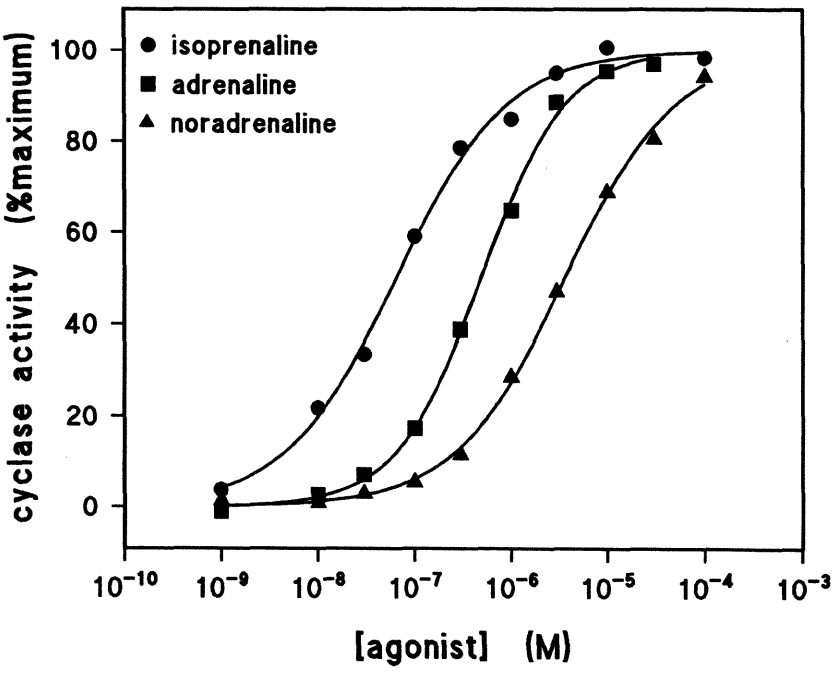


Fig 3.5 Rank order of potency of the 3 catecholamines isoprenaline, adrenaline and noradrenaline at stimulating adenylate cyclase in human right atrial membranes. Each curve represents a single experiment performed in triplicate. Specimen was from a patient not treated with β blockers. EC_{50} for isoprenaline = 64 nM, adrenaline = 490 nM and noradrenaline 3,600 nM

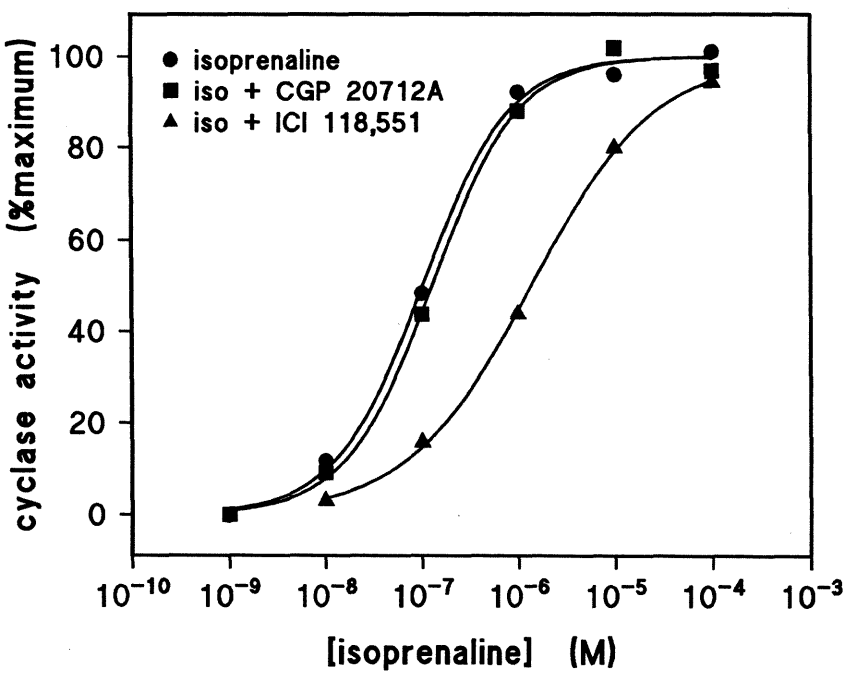


Fig 3.6 Effect of selective β_1 adrenoceptor blockade (100 nM CGP 20712A) and selective β_2 adrenoceptor blockade (50 nM ICI 118,551) on the dose response of isoprenaline stimulation of adenylate cyclase in human right atrium. EC_{50} in the absence of antagonists = 0.1 μM, in the presence of CGP 20712A = 0.13 μM and in the presence of ICI 118,551 = 1.4 μM. Single experiment performed in triplicate. Biopsy obtained from a patient not taking β blockers preoperatively.

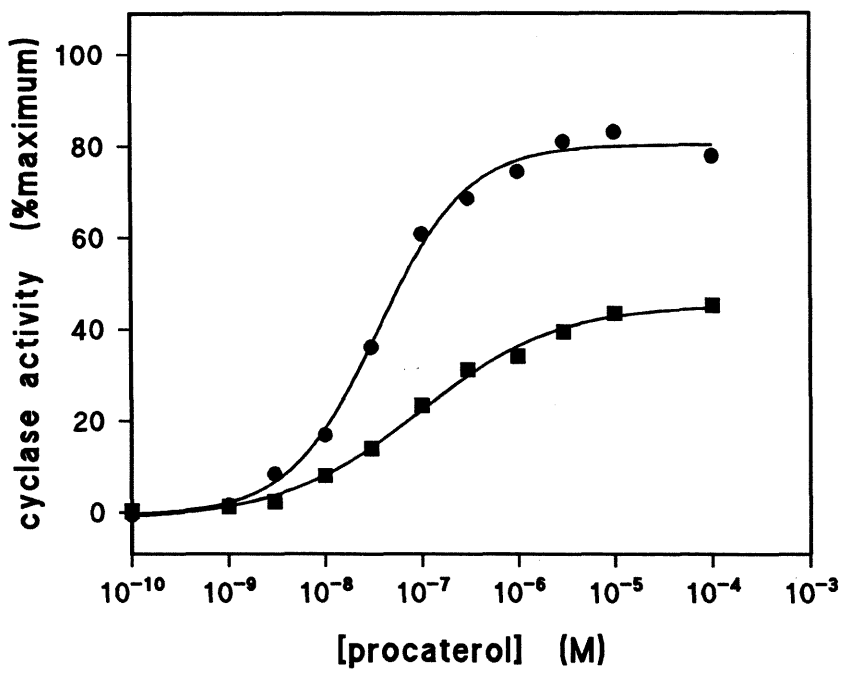


Fig 3.7 Dose response for the stimulation of adenylate cyclase by the β_2 selective partial agonist procaterol relative to maximal stimulation by isoprenaline. Each curve is the mean of at least 2 experiments performed in triplicate. ● right atrial membranes from a patient not taking β blockers, EC_{50} = 36 nM, intrinsic activity = 80% that of isoprenaline. ■ rat left ventricular membranes, EC_{50} = 94 nM, intrinsic activity = 45% that of isoprenaline.

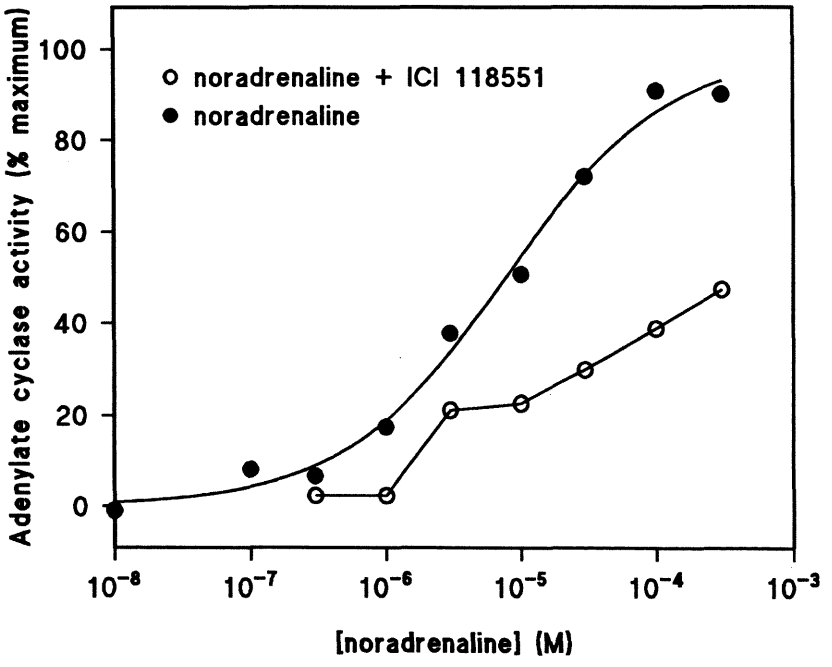


Fig 3.8 Effect of 100 nM ICI 118,551 on the dose response of noradrenaline stimulation of adenylate cyclase in human right atrial membranes. Single experiment performed in triplicate. There is a suggestion that low concentrations of the relatively β_1 adrenoceptor agonist noradrenaline are less sensitive to β_2 adrenoceptor blockade than higher concentrations, but this could not be reliably reproduced.

3.3.3 β adrenoceptor subtype coupling to adenylate cyclase in rat left ventricle

The rank order of potency of the 3 catecholamines at stimulating adenylate cyclase in membranes prepared from rat left ventricle was consistent with a predominately β_1 adrenoceptor mediated effect (fig 3.9). Inhibition of the adenylate cyclase stimulation by 1 μM isoprenaline with varying concentrations of CGP 20712A yielded a biphasic curve (fig 3.10). The majority of the adenylate cyclase stimulation was inhibited by low concentrations of this β_1 selective antagonist, suggesting that this was mediated by β_1 adrenoceptors. Computer assisted analysis using a 2 site model demonstrated that β_1 adrenoceptor mediated stimulation of adenylate cyclase by isoprenaline accounted for 67% of total stimulation. Procaterol had a lower intrinsic activity in membranes prepared from rat ventricle than from human atrium (fig 3.7) suggesting weaker coupling of the β_2 subtype in the rat heart.

3.3.3 Effect of forskolin on the stimulation of adenylate cyclase

Forskolin is poorly soluble in water and the strongest solution attainable in the Tris buffer was 30 μM. Fig 3.11 shows the dose response for forskolin stimulation of adenylate cyclase in rat left ventricular membranes. It would appear that the maximally effective concentration exceeds that which can be placed into solution. In subsequent experiments forskolin was used at a concentration of 20 μM, being the highest concentration that could be reliably achieved.

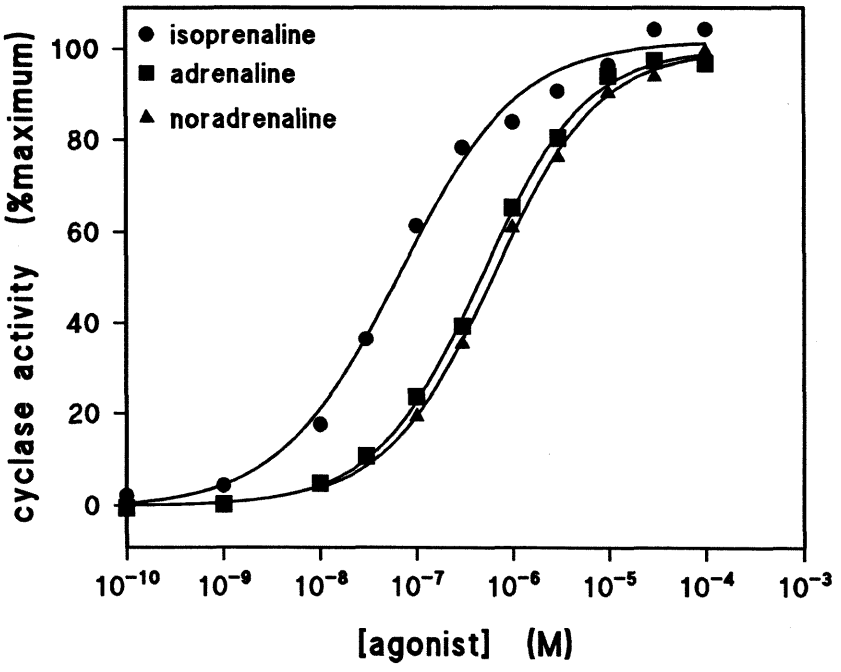


Fig 3.9 Rank order of potency of the 3 catecholamines isoprenaline, adrenaline and noradrenaline at stimulating adenylate cyclase in rat left ventricular membranes. Each curve represents the mean of 3 experiment performed in triplicate. EC_{50} for isoprenaline = 65 nM, adrenaline = 490 nM and noradrenaline 630 nM

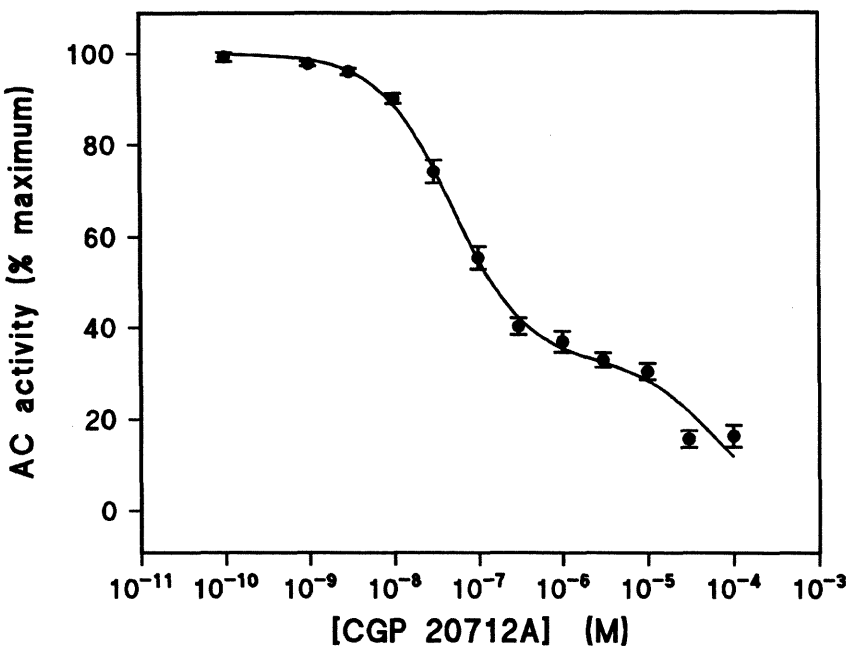


Fig 3.10 Effect of varying concentrations of CGP 20712A at inhibiting the stimulation of adenylate cyclase activity by 1 μM isoprenaline in rat ventricular membranes. Mean of 6 experiments performed in triplicate. 67% of total isoprenaline stimulation was inhibited by low concentrations of CGP compatible with inhibition of β_1 adrenoceptors.

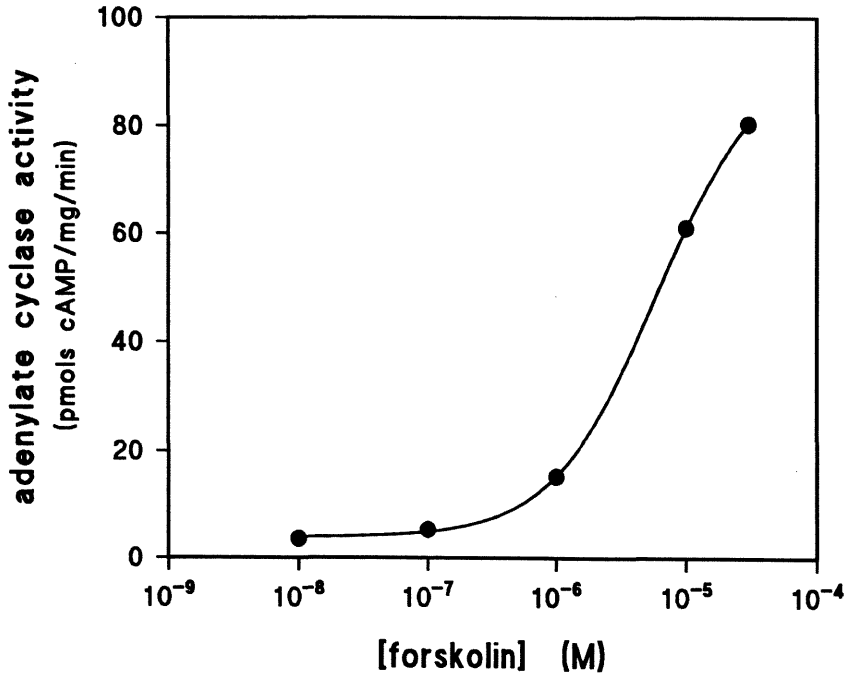


Fig 3.11 Dose response of forskolin stimulation of adenylate cyclase in rat ventricular membranes. EC_{50} = 5.9 μM.

3.4 Conclusions

The data presented in this chapter have confirmed previous reports that the coupling of human cardiac β adrenoceptors to adenylate cyclase is predominantly mediated via the β_2 subtype. It was not possible to reliably demonstrate coupling of β_1 adrenoceptors to adenylate cyclase in human right atrium.

In contrast, the majority of β adrenoceptor coupling in rat ventricle was mediated by the β_1 subtype. This has not previously been shown, but correlates well with functional studies demonstrating that the inotropic response in this species is exclusively mediated by the β_1 subtype. The rat model therefore compliments the human one and should enable drug induced changes in β_1 coupling to be studied.

Chapter 4

Human Study - design and clinical data

This chapter includes a description of the human study and its clinical results. The biochemical data will be presented in the next chapter.

4.1 Introduction

The study of human cardiac β adrenoceptors is limited by the availability of tissue since this can only be obtained at open heart surgery or by percutaneous biopsy. Neither procedure is performed in normal subjects. Thus to study the effect of a drug on human cardiac β adrenoceptors, a group of patients has to be found from whom cardiac tissue can be obtained and in whom there is an ethically acceptable indication for therapy with the drug in question.

Patients with coronary artery disease and angina pectoris that is inadequately controlled on medical therapy are usually considered for revascularisation with coronary artery bypass grafting (CABG) or percutaneous transluminal coronary angioplasty. As discussed in chapter 1, xamoterol has proven anti-ischaemic effects through its ability to limit heart rate response to exercise and possibly by improving diastolic function at rest. Patients with ischaemic heart disease undergoing CABG therefore represent a possible group to study the effects of this drug. The right atrial appendage, which is cannulated during CABG to provide venous blood for the cardiopulmonary bypass circuit, has been used in numerous previous studies as a source of myocardial tissue for biochemical and functional studies.

Most patients undergoing myocardial revascularisation are taking anti-anginal therapy. This will usually include a β-blocker in patients who can tolerate it.

It is now generally accepted that β-blockade should be continued until surgery to limit intra-operative ischaemia. However β-blocker therapy may be associated with an increased requirement for inotropic or intra-aortic balloon pump support in the early post-operative period (Jones *et al*, 1976; Weschler, 1980). It is possible that a partial agonist such as xamoterol, whilst still preventing intra-operative ischaemia, may reduce this requirement.

Atrial fibrillation or flutter occurs in the early postoperative period in 20-30% of patients undergoing CABG surgery (Frost *et al*, 1992) and pre-operative therapy with β adrenoceptor antagonists is an independent risk factor for this (Leitch *et al*, 1990). The risk of post-operative supraventricular tachycardia in patients treated pre-operatively with β-blockers is reduced by reinstituting therapy in the early post-operative period (Salazar *et al*, 1979; Silverman *et al*, 1982). These data, along with the known effects of β-blockers on β adrenoceptor sensitivity (see chapter 1), suggest that atrial dysrhythmias are a manifestation of β adrenoceptor hypersensitivity following antagonist withdrawal. It is not known whether therapy with xamoterol would be as effective as therapy with a full antagonist at preventing post-operative supraventricular tachycardias.

Thus patients undergoing CABG represent a useful model to examine not only the effect of xamoterol therapy on human cardiac β adrenoceptor regulation but also on the peri-operative course of such patients. Because most patients undergoing CABG are already taking β adrenoceptor antagonists, it was considered unethical to have a placebo control group as this would have entailed withdrawing β blockers in patients with severe ischaemic heart disease shortly before surgery. Standard therapy with atenolol was used for the control group, as there is already published data on

the effect of this agent on human right atrial β adrenoceptors (Golf and Hansson, 1986; Michel *et al*, 1988; Motomura *et al*, 1990).

4.1.1 Aim of Study

The principal aim of the study was to compare the effects of chronic therapy with xamoterol and atenolol on the regulation of β adrenoceptor subtype density and coupling to adenylate cyclase. The secondary aim was to compare the effect of these agents on the peri-operative course of patients undergoing CABG.

4.2 Methods

4.2.1 Trial Design

Randomised, double blind, between group study of xamoterol and atenolol therapy commencing at least 5 weeks prior to CABG and continuing for 6 weeks after operation.

4.2.2 Subjects

Patients with stable angina pectoris on the routine waiting list for CABG at Groby Road Hospital were considered for entry into the trial. Age greater than 70 years, child bearing potential in women, angina predominantly at rest or unstable angina, obstructive airways disease, overt heart failure, intermittent claudication, second or third degree heart block and serum creatinine greater than 250 μM were specific exclusion criteria. The study was passed by the local ethics committee and all patients gave informed written consent. Using data from Golf and Hansson (1986) it was estimated that 18 analysable biopsies would be required in each group to show a difference in β adrenoceptor density of 15 fmols/mg protein (approximately 15%) at the 5% significance level with 80% power. Allowing for withdrawals and non-analysable biopsies a total of 55 patients were recruited.

4.2.3 End points

Primary: β adrenoceptor subtype density and coupling to adenylate cyclase

Secondary: Frequency of angina attacks and sublingual GTN consumption

Adverse events

Peri-operative requirement for inotropes, diuretics and antiarrhythmic agents

4.2.4 Protocol

Visit 1. At least 5 weeks prior to surgery, patients were seen in the outpatient department. The nature of the study was explained and permission to participate sought. All routine pre-operative assessments were performed at this visit. Patients were requested to discontinue their usual β-blockers and randomised in a double blind fashion (using a double dummy technique) to receive either xamoterol 200 mg bd or atenolol 100 mg od in their place. Both are standard therapeutic dosages. All other concomitant therapy was noted and continued. Diaries to record daily attacks of angina and use of sublingual GTN were issued. Left ventricular ejection fractions were calculated from single plane left ventricular angiograms (right anterior oblique projection) performed at the time of diagnostic catheterisation. Coronary angiograms were reviewed by a radiologist and the extent of the disease quantified using the Green Lane scoring system (Brandt *et al*, 1977). This system scores myocardial perfusion according to the extent of an artery's stenosis and the amount of left ventricular myocardium which it supplies. A complete absence of perfusion would be represented by a maximum score of 15. An example of a report is given in appendix 4.

Visit 2. One week after visit 1 the patients attended for a safety check, Adverse events were recorded and a clinical examination was performed. A radionucleide MUGA scan was performed to provide a more contemporaneous assessment of left ventricular function as in most patients diagnostic catheterisation had been performed several months previously.

Visit 3. At least 4 weeks after visit 2 the patients were admitted to hospital for their surgery. If possible this was scheduled for 2 days before the operation. A clinical examination was performed and the angina diaries were collected. A holter monitor (Tracker, Reynolds Medical) was applied for 24 hour ambulatory ECG recording and subsequently analysed on a Reynolds Pathfinder 3 (Mk II) analyser. Medication was continued up to and including the morning of surgery. At operation any requirement for additional cardiovascular therapy was recorded. Immediately prior to instituting cardiopulmonary bypass, a biopsy of the right atrial appendage was taken. This was placed in ice cold 0.9% saline and transported immediately to the laboratory.

Visit 4 As soon as the patients tolerated oral therapy (usually the first post-operative day) the pre-operative randomised therapy was continued with an unchanged dose of xamoterol (200 mg bd) and a reduced dose of atenolol (25 mg bd). The dose of atenolol chosen was that routinely given by the cardiothoracic surgeons for prophylaxis against post-operative supraventricular tachycardias. For the first 5 post-operative days, resting heart rate and blood pressure was recorded at the same time each morning and any additional cardiovascular therapy (inotropes, diuretics and antiarrhythmics) or the occurrence of clinically significant arrhythmias was noted. 4 days post-operatively a further 24 hour ECG recording was performed. The fifth postoperative day marked the end of this visit.

Visit 5 Approximately 6 weeks following surgery the patients were reviewed in outpatients. Any adverse events were recorded and a clinical assessment performed. This visit marked the end of the trial and the study medication was discontinued.

4.2.5 Statistics

Patient numbers were compared with a χ^2 or Fisher exact test depending on sample size. Comparison between groups was performed with an unpaired t-test for normally distributed data and the Mann Whitney test for non-parametric data.

4.3 Results

Data will be presented in this chapter the form of summary tables. Individual patient data are presented in appendix 1.

4.3.1 Patient details

55 patients were recruited, of whom 28 were randomised to xamoterol and 27 to atenolol. 9 patients in the xamoterol group and 4 in the atenolol group withdrew pre-operatively (see below). In addition, analysable biopsies were not obtained in a further 2 patients in the atenolol group as a result of administrative problems in one and preparation problems in another. Thus 19 biopsies were obtained from 19 operations in the xamoterol group and 21 biopsies were obtained from 23 operations in the atenolol group.

On entry, the patients were evenly matched for sex, degree of ischaemia and left ventricular ejection fraction as determined by left ventricular angiography (table 4.1). The xamoterol group were slightly older ($p=0.04$, two sided t test).

Table 4.1 Characteristics of the 55 patients randomised to receive either xamoterol or atenolol. Values are means (sd)

	xamoterol (n=28)	**atenolol** (n=27)
Age (years)	57 (6)	53 (8)
Sex	25M, 3F	25M, 2F
2 vessel disease	6	6
3 vessel disease	22	21
Ischaemia score	11.2 (1.8)	11.3 (1.5)
Angiographic LVEF (%)	57 (11)	62 (9)

Patients withdrawn before visit 2 did not have a radionucleide MUGA scan. 24 patients in each group therefore had this study. The mean (sd) ejection fraction was 58 (8)% in the xamoterol group and 59 (7)% in the atenolol group (p = not significant). This compares with angiographic ejection fractions of 57 (11)% and 62 (7)% (p = not significant) in the same patients. This would suggest that there had been no significant deterioration in left ventricular function between angiography and the study and also that one week's treatment with either drug did not cause major changes in ejection fraction. The 2 methods of determining ejection fractions are compared in figure 4.1.

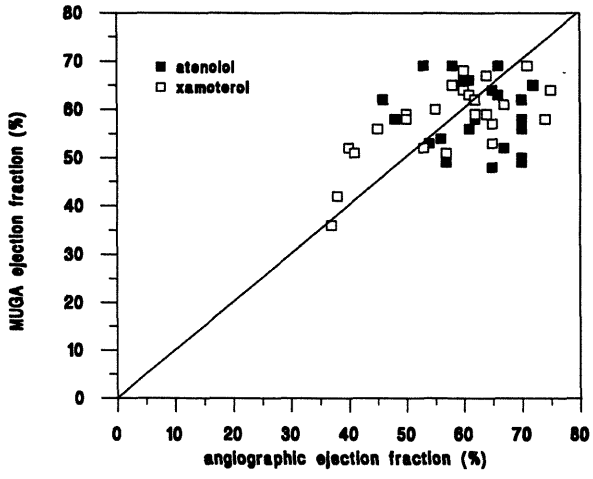


Fig 4.1 Comparison of 2 methods for determining left ventricular ejection fraction with line of identity

4.3.2 Drug therapy

Anti-anginal therapy on entry to the trial is detailed in table 4.2. Nearly all patients were taking β-blockers. This is not unexpected as the absence of contraindications to β-blockers was a prerequisite for entry into the trial. Fewer patients were taking calcium antagonists or long acting nitrates.

Table 4.2 Anti-anginal therapy on entry to trial. All therapy other than β-blockers were continued during the pre-operative phase

	Xamoterol (n=28)	**Atenolol** (n=27)
β blockers	24	24
Calcium antagonists	16	18
Oral or trans-dermal nitrates	15	12

4.3.3 Haemodynamic variables

13 patients were withdrawn prior to surgery and thus resting haemodynamic data was available for 42 patients both on entry and after 5 weeks therapy (table 4.3). There was no significant difference in resting heart rate or blood pressure on entry to the trial. However resting heart rate was significantly higher in the xamoterol group after 5 weeks therapy. This was associated with a significantly higher rate pressure product in the xamoterol group at visit 3, both compared to the atenolol group at the same visit ($p<0.0001$) and the xamoterol group at entry ($p<0.002$).

Table 4.3 Resting haemodynamic data (supine) for the 42 patients not withdrawn prior to surgery. BP = blood pressure, RPP = rate pressure product. Values are means (sd)

	Xamoterol n=19	**Atenolol** n=23	**p** (t test)
Entry pulse rate	59 (9)	59 (10)	ns
Entry systolic BP (mm Hg)	139 (20)	142 (19)	ns
Entry diastolic BP (mm Hg)	75 (10)	78 (8)	ns
Entry RPP	8182 (1629)	8418 (1874)	ns
Pre-op pulse rate	72 (8)	57 (8)	<0.0001
Pre-op systolic BP (mm Hg)	140 (19)	132 (17)	ns
Pre-op diastolic BP (mm Hg)	83 (15)	73 (7)	<0.02
Pre-op RPP	10109 (1857)	7555 (1131)	<0.0001

4.3.4 Angina diaries

30 patients returned the angina diaries. The first week was excluded from the analysis to avoid the effect of prior therapy. 2 patients returning diaries were withdrawn in the first week and their data thus excluded from analysis. Complete data on angina attacks were available in 15 xamoterol patients and

13 atenolol patients. 3 patients in each group failed to record their GTN consumption and were excluded from this part of the analysis. Thus complete data on GTN consumption was available in 12 xamoterol patients and 10 atenolol patients. It is possible that GTN consumption was not recorded because it was not used. Treating this as missing data would then have the effect of overestimating the overall consumption in each group.

Angina attack rate and GTN consumption were not normally distributed and a Mann Whitney test was used to compare the 2 groups. There was no significant difference in weekly angina attack rate or GTN consumption (table 4.4).

Table 4.4 Data from angina diaries. GTN refers to number of sublingual doses of GTN (tablets or aerosol). Values are medians (interquartile range)

	Xamoterol	Atenolol	p
Angina attacks/week	4.0 (1.8-8.8) (n=15)	5.8 (0.4-10.5) (n=13)	1.0
GTN/week	4.2 (0.6-21.5) (n=12)	5.6 (0.0-12.8) (n=10)	0.88

4.3.5 Adverse events

Table 4.5 lists all adverse events reported more than once in the pre-operative phase. Palpitations were observed exclusively in the xamoterol group and the difference was statistically significant. Otherwise there was no significant difference in the occurrence of adverse events between the 2 groups.

Table 4.5 Number of patients complaining of possible adverse events in the pre-operative period

Event	Xamoterol (n=27)	Atenolol (n=28)	p (Fisher Exact)
Worsening chest pain	8	5	ns
Palpitations	6	0	0.01
Fatigue/lethargy	1	3	ns
Headache	1	2	ns
Cold extremities	0	2	ns

One patient randomised to atenolol developed a myocardial infarct in the early post-operative period complicated by ventricular fibrillation and cardiogenic shock from which he died. This happened before he received any trial medication (9 hrs post-operatively). Another patient on atenolol perforated a colonic diverticulum and required an emergency laparotomy 3 days following surgery. Both events were thought to be unrelated to the trial medication.

4.3.6 Withdrawals

Reasons for withdrawal pre-operatively are given in table 4.6. There were no significant differences between the 2 treatment groups. The operation was postponed in 3 patients in the xamoterol group. In 2 of these patients the symptoms were on review thought to be not severe enough to require surgery and in the third patient the discovery of severe carotid artery disease was felt to unacceptably increase the risk of surgery in a patient with relatively mild symptoms.

Table 4.6 Reasons for withdrawal during the pre-operative period

Reason	Xamoterol (n=28)	Atenolol (n=27)	p (Fisher Exact)
Worse angina	5	3	ns
General malaise	1	1	ns
Operation postponed	3	0	ns

3 patients (all on atenolol) were withdrawn in the postoperative period. This included the patient who died and the patient who perforated a diverticulum. In addition, one patient developed recurrent angina 10 days after surgery and was prescribed unblinded atenolol for his symptoms.

4.3.7 Rhythm disturbances

Analysable tapes were obtained pre-operatively from 15 patients in the xamoterol treatment group and 19 in the atenolol group. Post-operatively analysable tapes were obtained from 18 patients in each group. The mean heart rate was higher in the xamoterol group both pre-operatively and 4 days post-operatively (fig 4.1). Median ventricular ectopic rates tended to be higher in the xamoterol treated patients (fig 4.2). To avoid making multiple comparisons of data, the hourly ectopic counts were summated to produce daily ectopic counts. When less than 24 hrs were recorded, the count was adjusted to give a calculated value for 24 hrs. The daily ectopic count was extremely variable, ranging from 0 to 8503 and not normally distributed (table 4.7). Comparison between groups was therefore made using the Mann Whitney test. Although the median ectopic count in 24 hrs was higher in the xamoterol group, the difference was not statistically significant.

Table 4.7 Median and interquartile ranges of ventricular ectopics occurring over 24 hours from pre and post-operative 24 hr holter monitoring.

	Xamoterol	Atenolol	p
Pre-operative	126 (14-780)	21 (10-165)	0.298
Post-operative	59 (4-464)	20 (8-94)	0.319

No patients had sustained ventricular or supraventricular tachycardias during the 2 periods of holter monitoring. Pre-operatively 4 patients had one or more episode of repetitive ventricular ectopics (3 or more consecutive beats) in the xamoterol group compared with none in the atenolol group (p=0.11, Fisher exact test). Post operatively one patient in each group had repetitive ventricular ectopics.

Only 1 patient (on xamoterol) developed atrial fibrillation post operatively. This developed on the third postoperative day and required therapy with digoxin, verapamil and amiodarone. His trial medication was continued and he subsequently reverted to sinus rhythm. As described above, one patient (randomised to atenolol) developed ventricular fibrillation 9 hours after surgery due to an acute myocardial infarct and subsequently died of cardiogenic shock.

4.3.8 Requirements for additional cardiovascular therapy

During surgery

Blood pressure under anaesthesia (when not on cardiopulmonary bypass) was carefully controlled by the anaesthetists predominately through changes

in fluid replacement. Atropine was given for excessive bradycardia in 6 patients in the atenolol group and only 1 in the xamoterol group (p=0.08, Fisher's exact test).

Post operatively

In the early postoperative phase 6 patients in the atenolol group and 1 patient in the xamoterol group (p=0.08, Fisher's exact test) required inotrope support. All patients received diuretics post-operatively. The mean (± SEM) daily requirement (mg of frusemide) was higher in the atenolol group (50.3 ± 4.5 vs 36.3 ± 4.3; p<0.03, two sided t test).

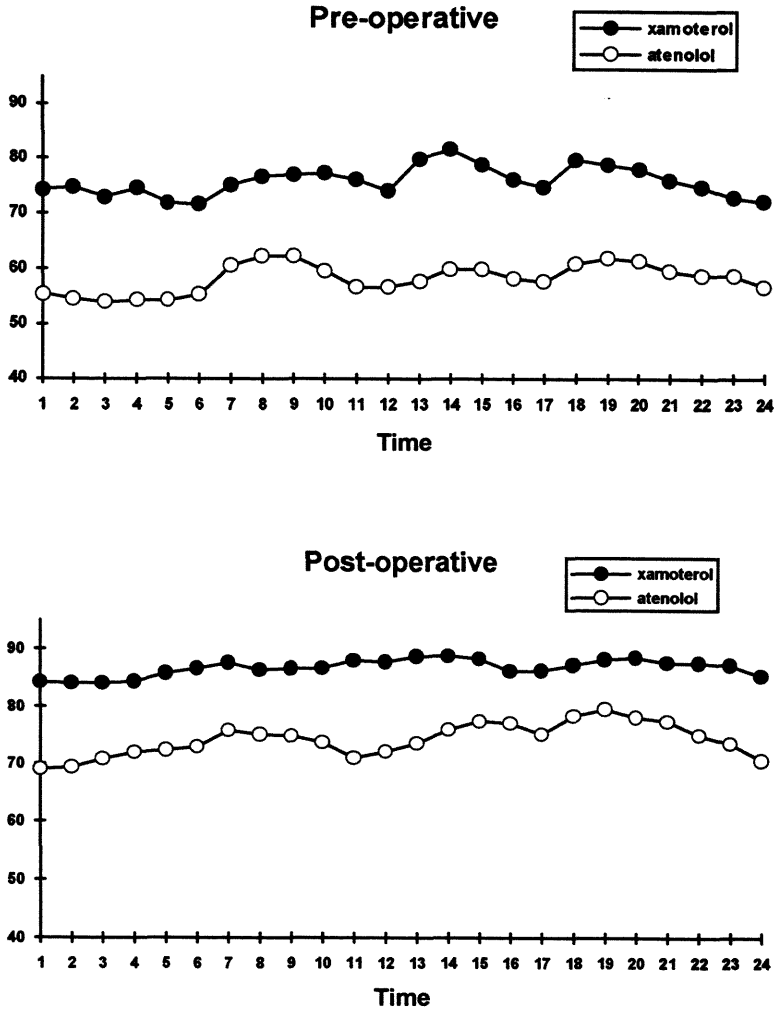


Fig 4.1 Graphs of mean heart rate over 24 hrs determined from the pre- and post-operative holter monitoring

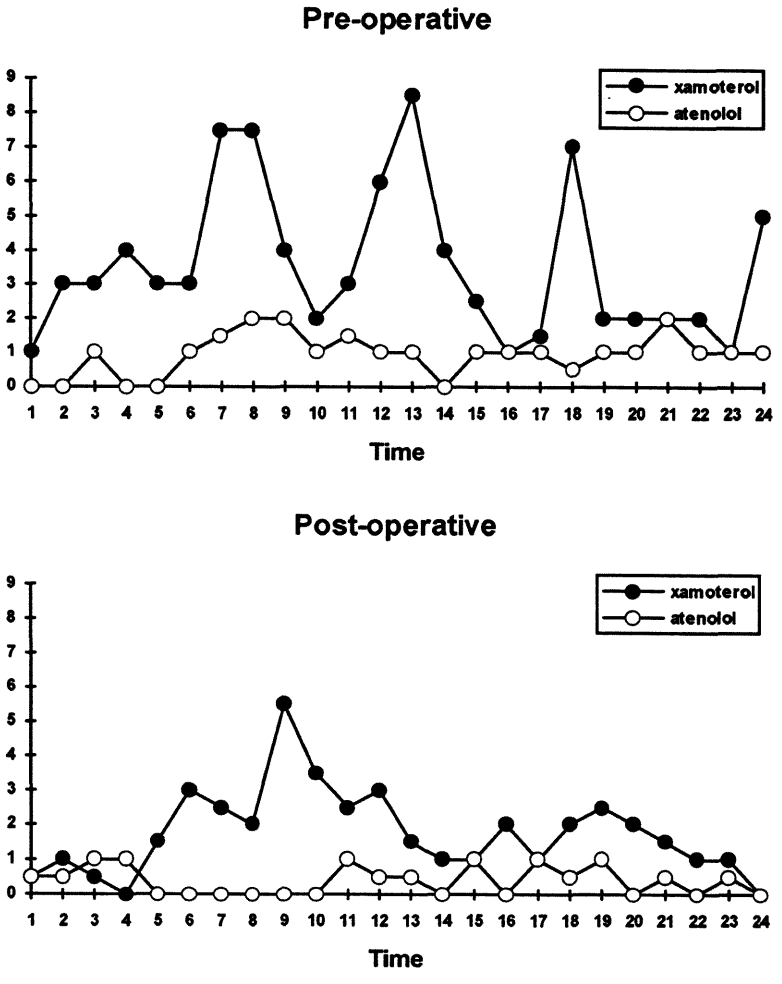


Fig 4.2 Graphs of median hourly ventricular ectopic rate over 24 hrs determined from the pre- and post-operative holter monitoring

4.4 Conclusions

The primary aim of this study was to investigate the effect of xamoterol on the regulation of cardiac β adrenoceptors. However a considerable amount of clinical information has been obtained on the use of xamoterol in ischaemic heart disease and its effect on the peri-operative course of patients undergoing CABG surgery.

Xamoterol has previously been shown to have anti-anginal properties when compared with placebo (Barrios *et al*, 1986; Detry *et al*, 1984; Molajo *et al*, 1987) but has not been directly compared with other anti-anginal therapy. In this study, patients with severe ischaemic heart disease were randomised in a double blind, prospective fashion to receive either xamoterol or standard therapy with the full antagonist atenolol. There was no significant excess of patients developing, or withdrawing from the study because of, worsening angina in the xamoterol group when compared to atenolol. Furthermore there was no significant difference in weekly attacks of angina or GTN consumption between groups. Although the diary data is incomplete there is no reason to suggest a systematic bias to favour one of the treatment groups. In particular there was no significant difference in the rate of return of diaries (17 from 28 patients in the xamoterol group and 13 from 27 patients in the atenolol group). Thus the evidence from this study would suggest that xamoterol has a similar efficacy to atenolol in the control of the symptoms angina.

Xamoterol has 2 properties that might explain its anti-anginal activity (see table 2, chapter 1). Firstly it has been demonstrated both in normal volunteers and patients with ischaemic heart disease that xamoterol limits the heart rate response and rise in rate pressure product on exercise. Secondly xamoterol improves the rate of left ventricular relaxation at rest and by

reducing end diastolic pressure this might also improve coronary perfusion. Exercise testing was not included in the protocol. However, mean hourly heart rate in the xamoterol group exceeded that in the atenolol group throughout the 24 hour period of holter monitoring (fig 4.2). After 5 weeks therapy resting heart rate and rate pressure product were significantly higher in the xamoterol group than the atenolol group. This would suggest a higher myocardial oxygen consumption, at least at rest, on xamoterol. Patients with angina predominantly at rest were excluded from the study and it is conceivable that such patients would not have tolerated xamoterol. It is also possible that xamoterol's effect on diastolic function may have contributed to its anti-anginal properties.

The data presented here do not necessarily imply that xamoterol increases resting heart rate or rate pressure product compared to placebo since the comparison was against a full antagonist. The increases seen over the values at entry in the xamoterol predominantly reflect the fact that the vast majority of patients were already on β blockers and consequently had a low resting heart rate. Placebo controlled studies have shown that xamoterol has a variable effect on resting heart rate and blood pressure (see chapter 1, table 2).

It is well recognised that catecholamines may increase ventricular irritability (Lubbe *et al*, 1992). Previous studies with xamoterol have failed to show a significant increase in ventricular ectopic activity in patients with heart failure (The xamoterol in severe heart failure group, 1990; Virk *et al*, 1990). Indeed xamoterol has been shown to be of use in the management of patients with drug resistant ventricular tachycardia (Paul *et al*, 1989; Bashir *et al*, 1992). Although there was a trend towards more ventricular ectopics in the xamoterol group, this did not reach statistical significance. Even in the

xamoterol group the median ventricular ectopic count in 24 hrs was 126, equivalent to just over 5 per hour. Again it should be noted that the comparison was against a full antagonist which might be expected to reduce the number of ventricular ectopics.

Supraventricular arrhythmias, in particular atrial fibrillation, are common in the early post-operative period following coronary artery bypass surgery. The reported incidence varies from 20-30% depending in part on the rigorousness of the assessment (Frost *et al*, 1992). Multivariate analysis reveals 2 factors which increase the relative risk of developing post-operative atrial fibrillation - advancing age and pre-operative treatment with β adrenoceptor antagonists. The latter is thought to be a manifestation of a β adrenoceptor hypersensitivity following antagonist withdrawal. The risk in such patients can be significantly reduced by post-operative therapy with β-blockers. In this study only 1 of the 42 patients undergoing surgery developed clinically apparent atrial fibrillation in the early post-operative period giving an overall incidence of 2.4%. There was no significant difference in the incidence of atrial fibrillation in the xamoterol group (1 of 19) compared to the atenolol group (0 of 23) using the Fisher exact test. There are 2 possible explanations. Firstly pre-operative therapy with xamoterol may not predispose patients to develop post-operative atrial fibrillation. Alternatively post-operative therapy with xamoterol may be as effective in preventing atrial fibrillation as atenolol. The effect of xamoterol on β adrenoceptor sensitivity will be discussed more thoroughly in the following 2 chapters.

Peri- and post-operative therapy with xamoterol may have potential advantages over a full antagonist. Full antagonists may cause significant brady-arrhythmias and negative inotropy. Fewer patients receiving xamoterol required atropine during surgery or positive inotropes post-operatively though

the differences did not reach statistical significance. Total diuretic requirements were significantly lower during the first 6 post-operative days in the patients receiving xamoterol.

Xamoterol was well tolerated in these patients with severe coronary artery disease. There was no significant difference in the numbers of patients withdrawing with possible adverse events (6 of 28 on xamoterol and 4 of 27 on atenolol). Adverse events were uncommon in either group with the exception of palpitations which only occurred in the xamoterol group (6 patients). This was a statistically significant difference.

The final conclusions from the clinical aspects of this study must take into account the small sample size and the selected nature of the patients. In particular patients had stable angina pectoris and no contraindications to therapy with β adrenoceptor antagonists. With these provisos, xamoterol was well tolerated and had a similar efficacy (as assessed by symptoms) to atenolol in patients with angina pectoris and severe ischaemic heart disease. Palpitations were more frequent and there was an insignificant trend to greater ventricular ectopic activity in the patients treated with xamoterol compared to atenolol. There was a trend for patients treated with xamoterol to require less atropine per-operatively and inotropes post-operatively and diuretic requirement was significantly reduced post-operatively. Xamoterol therapy did not increase the incidence of post-operative atrial fibrillation or flutter. Thus therapy with the partial agonist xamoterol may have some clinical benefits on the peri-operative course of patients undergoing CABG surgery when compared to the full antagonist atenolol.

CHAPTER 5

Human Study - biochemical aspects

5.1 Introduction

The design and clinical aspects of the human study have been described in chapter 4. The biochemical results will be discussed in this chapter.

5.1.1 Aim of Study

The aim of this study was to investigate the effect of chronic therapy with xamoterol on β adrenoceptor subtype density and coupling to adenylate cyclase in human right atrium.

5.2 Methods

5.2.1 Tissue procurement and preparation

Randomised therapy was continued up to and including the morning of surgery. At operation, a biopsy (weighing between 103 and 1034 mg) of the right atrial appendage was taken immediately prior to inserting the venous cannula into the right atrium to provide venous blood for the cardiopulmonary bypass circuit. The biopsy was placed in ice cold 0.9% saline and transported immediately to the laboratory. Crude membranes were prepared as described in chapter 2. These were then divided into aliquots, frozen and stored at minus 70°C. The median (interquartile range) time from last dose of medication to specimen removal was 4.2 (4.1-7.8) hr in the xamoterol group and 4.5 (4.0-7.8) hr in the atenolol group (p=0.84, Mann-Whitney test).

Of the 55 patients randomised in the trial, 13 were withdrawn prior to surgery as described in the preceding chapter. Analysable biopsies were not obtained in a further 2 patients because of administrative problems in one patient and technical problems in the other. Right atrial biopsies were therefore obtained from 40 randomised patients (19 randomised to xamoterol and 21 to atenolol). In addition, atrial biopsies were obtained from a further 5 patients not randomised to the trial who, at the time of surgery, were not taking β-blockers for reasons other than the presence of heart failure. All 5 were male with a mean (sd) age of 61 (5) years. 4 were taking calcium antagonists. Data from this group is included in the results for comparison, but since it is of small size and the patients were not prospectively randomised, direct statistical comparison with the treatment groups will not be performed. All trial specimens were analysed blind as to therapy.

5.2.2 Receptor binding studies

Total β adrenoceptor density was determined by Scatchard analysis of saturation binding experiments (performed in duplicate) of [^{125}I]iodopindolol binding as described in chapter 2. In view of the limited availability of tissue, subtype densities were determined by performing saturation experiments in the presence and absence of 100 nM CGP 20712A rather than using full displacement curves.

5.2.3 Adenylate cyclase assays

Assays were performed in triplicate as described in chapter 3. Since it proved impossible to reliably demonstrate coupling of β_1 adrenoceptors to adenylate cyclase, an assessment of total and β_2 adrenoceptor mediated

coupling was made. Total receptor mediated stimulation of adenylate cyclase was determined by estimating the response to a maximally effective concentration (10 μM) of the non-selective β adrenoceptor agonist isoprenaline. β_2 adrenoceptor mediated coupling was compared by estimating the intrinsic activity of a maximally effective concentration (10 μM) of the β_2 selective partial agonist procaterol relative to maximal stimulation with the full agonist isoprenaline. The intrinsic activity of a partial agonist will reflect the strength of coupling, increasing with improved coupling. This is likely to be more reliably observed than small shifts in dose response curves. Changes in G-protein function were determined by estimating basal adenylate cyclase activity in the presence and absence of 0.1 mM GTP. Whilst not a direct measure of individual G protein quantity or function, this does give an measure of the net *functional* consequences of such changes. Finally non-receptor mediated stimulation of adenylate cyclase was determined by estimating the response to 20 μM forskolin.

5.2.4 Statistics

Data are given as means with standard deviations (sd) or standard errors of the mean (sem) unless otherwise stated. Significance tests for continuous variables between treatment groups were made using the unpaired t-test for normally distributed data and Mann Whitney test for non-parametric data. The χ^2 or Fisher exact test was used to analyse proportions in the patient characteristics. A p value of less than 0.05 was taken as significant.

5.3 Results

Data are reported here in the form of summary tables. Individual patient data will be found in appendix 2.

5.3.1 Patient characteristics

Patient data for the 55 patients randomised have been summarised in chapter 4. Table 5.1 lists the characteristics of the 21 patients randomised to atenolol and 19 patients randomised to xamoterol from whom analysable biopsies were obtained. There was no significant difference in age, sex, left ventricular ejection fraction (either from angiography at the time of initial investigation or MUGA scan after 1 week of therapy), ischaemia score or concomitant therapy with calcium antagonists between groups.

Table 5.1 Characteristics of the patients from whom analysable biopsies were obtained. LVEF = left ventricular ejection fraction. Data are given as patient numbers or mean (sd)

	Xamoterol (n=19)	**Atenolol** (n=21)
Age (years)	57 (7)	55 (7)
Sex (male:female)	18M:1F	19M:2F
LVEF-angio (%)	57 (11)	61 (7)
LVEF-MUGA (%)	58 (7)	60 (6)
Ischaemia score	11.4 (2.0)	11.2 (1.5)
Ca antagonists (n)	12	15

5.3.2 Effect of treatment on β adrenoceptor density

There was no significant difference in the density of total, β_1 or β_2 adrenoceptors between the 2 treatment groups (table 5.2, table 5.3 and fig 5.1). Total and β_1, but not β_2, receptor density appeared higher in the treatment groups than in the comparator group on neither drug. There was no difference in the affinity of β adrenoceptors for [^{125}I]iodopindolol in the 2 treatment groups (Kd xamoterol group = 29.61 ± 2.44 pM, atenolol group = 29.99 ± 2.12 pM). β_1 adrenoceptors made up 68.4 ± 0.7% of total β adrenoceptors in the xamoterol group and 67.0 ± 0.7% in the atenolol group.

Table 5.2 Total and subtype β adrenoceptor density (fmols/mg protein). Data are given as means (sem). "No drug" refers to small comparator group of patients taking neither drug prior to surgery (see methods).

	Xamoterol (n=19)	**Atenolol** (n=21)	**No drug** (n=5)
Total	63.37 (2.37)	65.90 (2.93)	59.34 (2.81)
β_1	43.39 (1.78)	44.20 (2.03)	37.77 (1.84)
β_2	19.98 (0.79)	21.70 (1.06)	21.58 (2.07)

Table 5.3 Percentage difference of receptor density in xamoterol treated patients from those of atenolol treated patients with 95% confidence intervals (CI).

	% difference	**95% CI**	**p**
Total	-3.4%	-15.5, +7.7	0.51
β_1	-1.8%	-14.3, +10.6	0.77
β_2	-7.9%	-20.3, +5.1	0.20

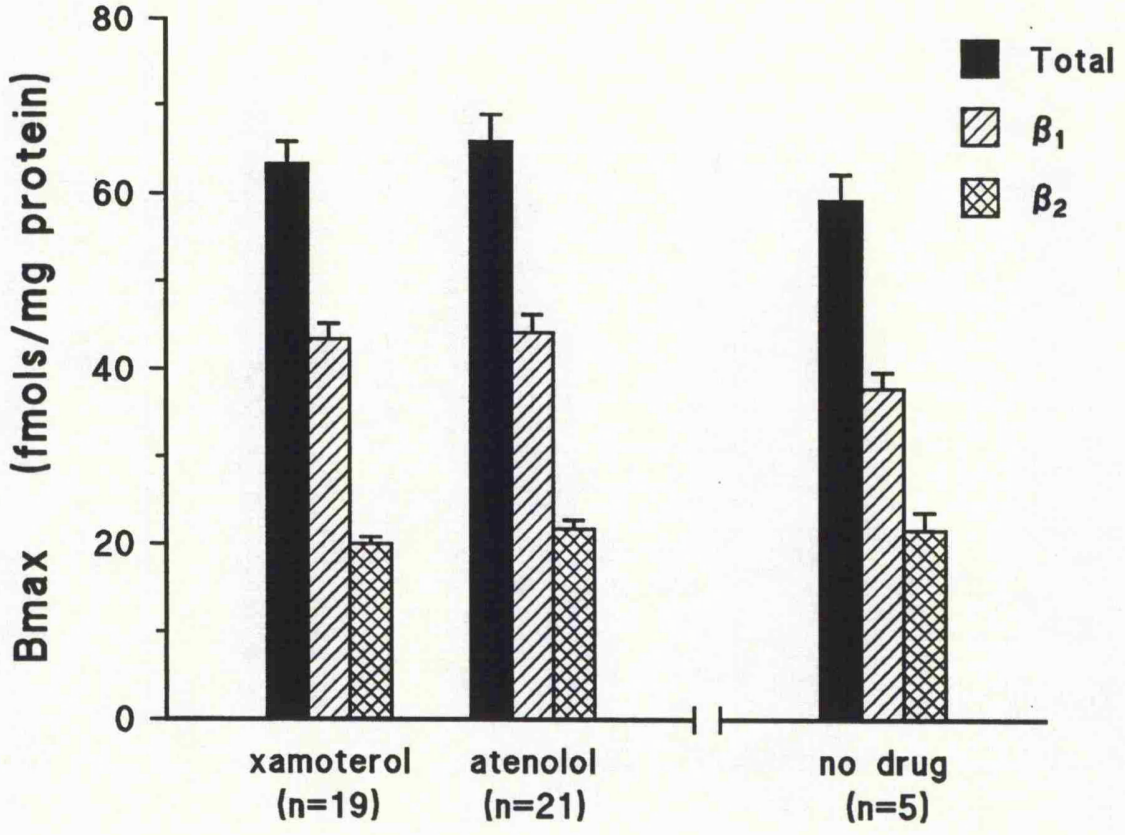


Fig 5.1 Total and subtype β adrenoceptor densities in right atrial biopsies by treatment group. "No drug" refers to the small group of 5 patients not randomised to the trial who were taking neither xamoterol or β-blockers at time of surgery.

5.3.3 Effect of therapy on adenylate cyclase activity

Basal and stimulated adenylate cyclase activities were significantly lower in the patients treated with xamoterol than in those treated with atenolol (table 5.4 and fig 5.2). This applied to receptor mediated stimulation (isoprenaline and procaterol) and receptor independent stimulation (GTP and forskolin). The values in the comparator group of patients taking neither drug were lower than those in either treatment group.

Table 5.4 Basal and stimulated adenylate cyclase activities (pmoles cAMP/mg protein/min). [a]p=0.0017, [b]p=0.0002, [c]p=0.0001 compared to atenolol. Data given as means (sem)

	xamoterol (n=19)	**atenolol** (n=21)	**no drug** (n=5)
basal (no GTP)	2.37 (0.17) [b]	3.52 (0.22)	1.75 (0.13)
basal (with GTP)	9.59 (0.72) [a]	12.84 (0.64)	6.14 (0.78)
isoprenaline 10μM	64.82 (3.82) [c]	90.37 (4.25)	44.94 (2.87)
procaterol 10μM	52.22 (3.38) [c]	75.73 (3.96)	35.02 (2.02)
forskolin 20μM	210.6 (15.2) [c]	307.8 (16.4)	138.1 (10.5)

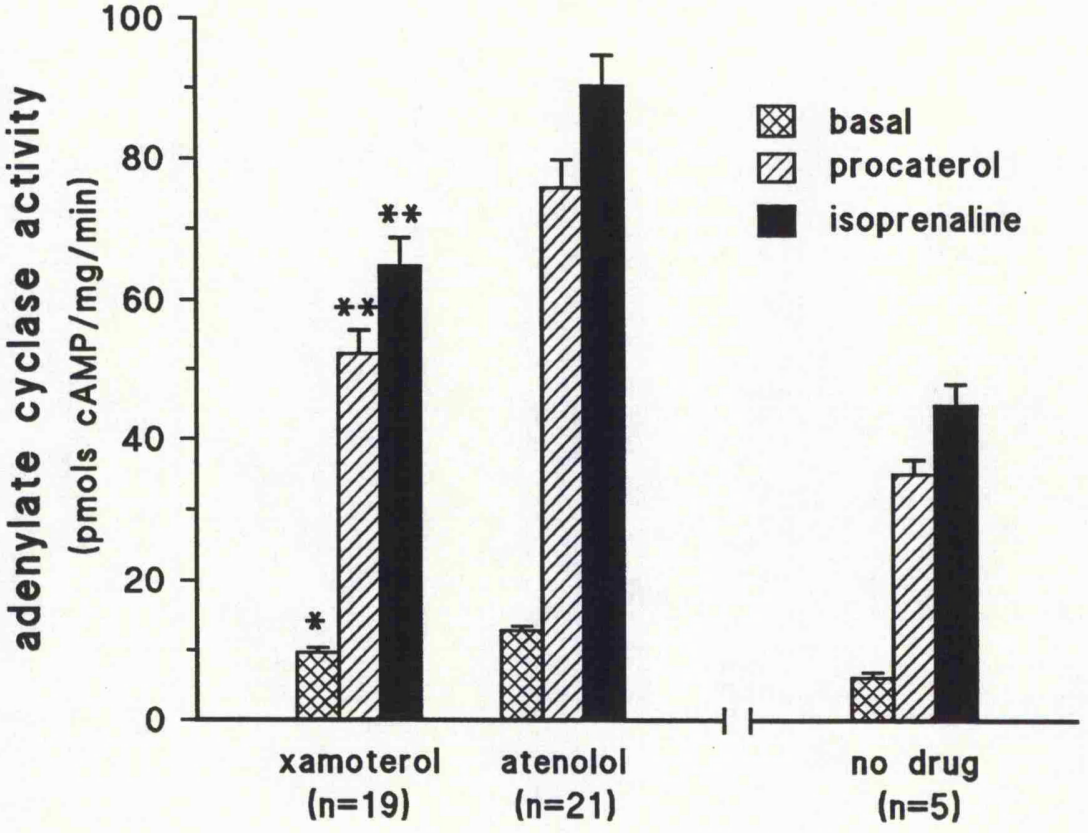


Fig 5.2 Bar chart of adenylate cyclase activity by treatment group under basal conditions (in the presence of 0.1 mM GTP) and following stimulation with 10 μM procaterol or 10 μM isoprenaline. *p<0.002, **p<0.0005 compared to atenolol group.

5.3.4 Effect of therapy on coupling of β_2 adrenoceptors to adenylate cyclase

The intrinsic activity of the β_2 adrenoceptor partial agonist procaterol (relative to that of isoprenaline) at stimulating adenylate cyclase was significantly greater in the group of patients treated with atenolol than in those treated with xamoterol (table 5.5). This would suggest enhanced coupling of the β_2 subtype to adenylate cyclase in the atenolol group relative to the xamoterol group. The intrinsic activity of procaterol was similar in the group of patients randomised to xamoterol and in the small comparator group of patients not taking β-blockers at the time of surgery suggesting that xamoterol therapy did not uncouple β_2 adrenoceptors.

Table 5.5 Intrinsic activity (%) of procaterol stimulation of adenylate cyclase relative to that of isoprenaline at stimulating adenylate cyclase. Data given as mean (sem). [a]$p<0.03$ compared to atenolol

	xamoterol (n=19)	**atenolol** (n=21)	**no drug** (n=5)
intrinsic activity	76.7 (1.1) [a]	80.6 (1.4)	75.2 (3.8)

5.3.5 Effect of therapy on isoprenaline stimulation of adenylate cyclase

To exclude the possibility that retained drug in the treatment groups was interfering with receptor mediated stimulation of adenylate cyclase, isoprenaline dose response curves were constructed from 3 patients chosen at random from the xamoterol, atenolol and comparator groups. There was no significant difference in the EC_{50} for isoprenaline stimulation. If anything, there appeared to be a trend for isoprenaline to be more potent in the 2 treatment groups (table 5.6 , fig 5.3).

Table 5.6 EC_{50} for isoprenaline stimulation of adenylate cyclase in membranes from 3 patients in each treatment group. Data given as mean (range)

	xamoterol (n=3)	**atenolol** (n=3)	**no drug** (n=3)
EC_{50} (μM)	0.65 (0.43-1.04)	0.71 (0.42-0.93)	1.10 (1.02-1.16)

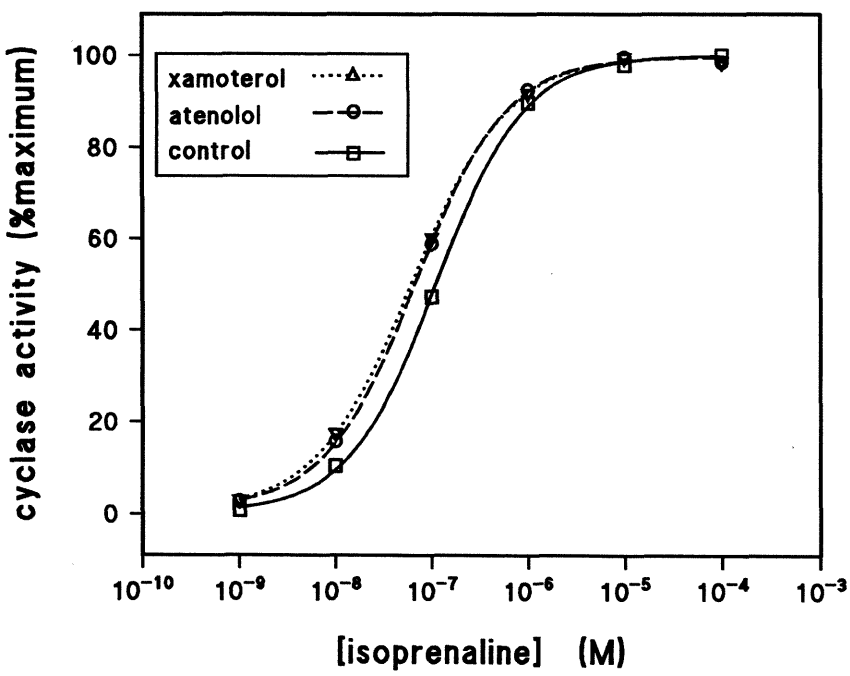


Fig 5.3 Dose response for isoprenaline stimulation of adenylate cyclase following therapy with xamoterol, atenolol or neither drug. Each point is the mean of 3 experiments, each using different specimens and performed in triplicate. EC_{50} xamoterol group = 0.65 μM, atenolol group = 0.71 μM and no drug = 1.10 μM

5.3.6 Effect of concomitant therapy with calcium antagonists on receptor density and coupling to adenylate cyclase

It has previously been suggested that therapy with calcium antagonists may affect β adrenoceptor density. A subgroup analysis was therefore made of receptor density and adenylate cyclase activity in patients who were or were not taking calcium antagonists.

When all trial patients were considered, β adrenoceptor densities tended to be lower in the subgroup of patients who were also taking calcium antagonists, significantly so for total and β_2 adrenoceptors (table 5.7). When treatment groups were analysed separately, the difference was more marked for those treated with xamoterol than atenolol, with significantly lower densities of total, β_1 and β_2 adrenoceptors in those patients also treated with calcium antagonists (fig 5.4). The difference was not significant in those treated with atenolol. This would suggest a possible interaction between calcium antagonists and xamoterol leading to a lower density of β adrenoceptors than that found in patients treated with xamoterol alone.

Since adenylate cyclase activities were different between the 2 treatment groups, it was not possible to combine treatment groups. However the effect of concomitant therapy with calcium antagonists on adenylate cyclase activity was determined within treatment groups (table 5.8). This revealed an insignificant trend toward lower basal and stimulated adenylate cyclase activities in those patients treated with calcium antagonists in both treatment groups.

Table 5.7 Effect of concomitant therapy with calcium antagonists on total and subtype β adrenoceptor density (fmols/mg protein). "Yes" = concomitant therapy with calcium antagonists, "no" = no concomitant therapy with calcium antagonists. Data given as mean (sem). [a]p<0.05 compared to all/no Ca antagonist, [b]p<0.03, [c]p<0.01 compared to xamoterol/no Ca antagonist.

	all		**xamoterol**		**atenolol**	
	yes (n=27)	no (n=13)	yes (n=12)	no (n=7)	yes (n=15)	no (n=6)
total	62.51[a] (2.53)	69.24 (2.11)	59.24[c] (3.22)	70.16 (1.06)	64.99 (3.74)	68.16 (4.60)
β_1	42.55 (1.78)	46.44 (1.69)	40.95[b] (2.54)	47.58 (0.92)	43.84 (2.52)	45.11 (3.61)
β_2	19.96[a] (0.89)	22.80 (0.77)	18.46[c] (0.85)	22.58 (0.99)	21.16 (1.40)	23.05 (1.30)

Table 5.8 Effect of concomitant therapy with calcium antagonists on basal and stimulated adenylate cyclase activity (pmoles cAMP/mg/min). "yes" = concomitant therapy with calcium antagonists, "no" = no concomitant therapy with calcium antagonists. Data given as means (sem)

	xamoterol		**atenolol**	
	yes (n=12)	no (n=7)	yes (n=15)	no (n=6)
basal (with GTP)	9.04 (0.83)	10.53 (1.34)	12.27 (0.65)	14.26 (1.46)
procaterol (10 μM)	48.93 (4.50)	57.85 (4.59)	73.27 (4.86)	81.88 (6.59)
isoprenaline (10 μM)	60.83 (4.86)	71.67 (5.69)	87.93 (5.31)	96.47 (6.75)
forskolin (20 μM)	194.6 (17.1)	238.1 (27.7)	294.1 (18.3)	342.0 (33.1)

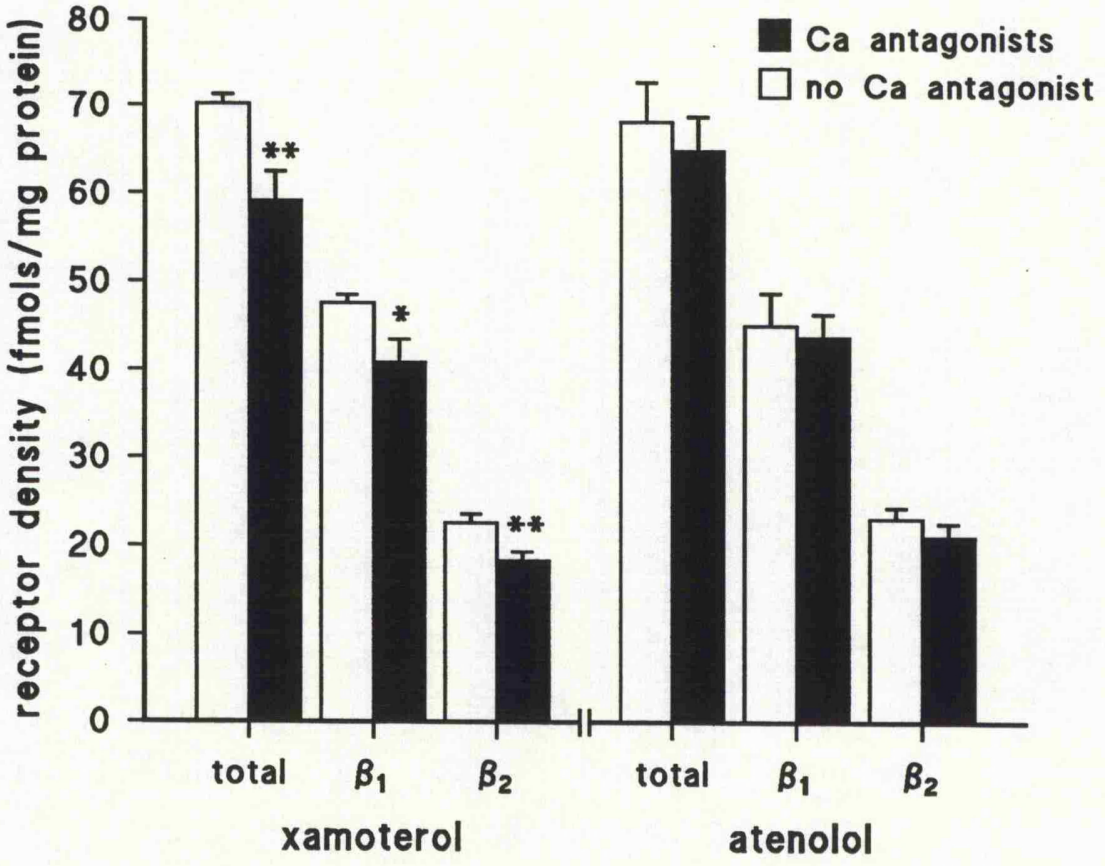


Fig 5.4 Effect of concomitant therapy with calcium antagonists on right atrial β adrenoceptor density in the 2 randomised treatment groups. *p<0.03, **p<0.01 compared to no therapy with calcium antagonists

5.4 Conclusions

This study was designed to investigate the effect of chronic therapy with the β_1 adrenoceptor partial agonist xamoterol on human cardiac β adrenoceptor subtype density and coupling to adenylate cyclase. A prospectively randomised controlled trial was considered essential to produce two groups that were well matched for variables that might affect β adrenoceptor density. Table 5.1 shows that this was successfully achieved with both groups well matched for age, left ventricular function, degree of ischaemia and concomitant therapy with calcium antagonists. As discussed in chapter 4, ethical constraints prevented the inclusion of a placebo control group and standard therapy with atenolol was used for a control group. Atenolol was chosen as there is already published data on this drug's effect on human right atrial β adrenoceptors (Golf and Hansson, 1986; Michel *et al*, 1988; Motomura *et al*, 1990). In addition, data has been included for comparison from a small group of 5 patients not randomised to the trial who were not taking β-blockers at the time of surgery for reasons other than overt heart failure. Direct statistical comparison between this group and the 2 treatment groups was not made because of the small size and unrandomised nature.

The study was designed to have the power to determine a 15% difference in receptor densities. Values less than this in the absence of changes in receptor coupling would be unlikely to have physiological significance. The 95% confidence limits on differences in receptor densities (table 5.3) show that the study met the intended power requirements. Thus there was no significant difference in total or subtype receptor densities between the 2 treatment groups. β_1 selective antagonists including atenolol have previously been shown to upregulate right atrial β adrenoceptors, with selective increases in the β_1 subtype. Michel *et al* (1988) showed significant increases

in total (37%) and β_1 (42%) adrenoceptors in patients treated with atenolol compared to those not treated with β blockers. Motomura *et al* (1990) showed a 44% increase in total β adrenoceptors in patients treated with various β_1 selective antagonists. In a smaller group of patients, Golf and Hansson (1986) showed a 10% increase in total receptors in those previously treated with atenolol. The changes in the first 2 mentioned studies are considerably greater than the 95% confidence limits of the difference between the 2 treatment groups in this study. By implication xamoterol therapy does not downregulate human cardiac β adrenoceptors and it is quite probable that it upregulates β_1 and total β adrenoceptors. Certainly total and β_1 adrenoceptor density in both treatment groups were higher than those in the small comparator group on no β blockers (table 4.2).

A fortuitous advantage of there being no difference in the density of receptors in the 2 treatment groups is that any differences in adenylate cyclase activity must result from changes in the signalling pathway distal to the receptor. Receptor mediated stimulation of adenylate cyclase by isoprenaline was 28% lower, and by procaterol 31% lower, in the xamoterol treated group when compared to the atenolol group (table 5.4). Retained drugs were unlikely to be interfering with receptor mediated stimulation of adenylate cyclase for 2 reasons. Firstly there was no significant difference in the affinity of receptors for [^{125}I]iodopindolol between the 2 treatment groups. Secondly there was no significant difference in the potency of isoprenaline at stimulating adenylate cyclase. If anything there was a trend for isoprenaline to be more potent in the treatment groups than in the small comparator group of patients taking neither drug (table 5.6).

The intrinsic activity of the β_2 selective partial agonist procaterol was significantly greater in the atenolol treated group (table 5.6). This would

suggest relatively enhanced coupling of the β_2 subtype to adenylate cyclase in patients pretreated with a full β_1 antagonist when compared to those pretreated with a β_1 partial agonist. Since the majority of coupling in this preparation is mediated via this subtype (see chapter 3), much of the difference in isoprenaline stimulation of adenylate cyclase is likely also to reflect changes in β_2 coupling.

Golf and Hansson (1986) noted that prior therapy with the β_1 selective antagonists atenolol and metoprolol resulted in a higher ratio of terbutaline (β_2 partial agonist) to isoprenaline stimulation of adenylate cyclase in human right atrium when compared to patients not treated with β blockers. Motomura noted enhanced isoprenaline stimulation of adenylate cyclase in patients pretreated with atenolol, metoprolol or bisoprolol relative to patients not treated with β blockers (Motomura *et al*, 1990). In the current study the comparison is between a β_1 selective antagonist, atenolol, and a β_1 selective partial agonist, xamoterol. A major strength of this study is that the patients were randomised prospectively to receive the 2 medications resulting in patients who were well matched for all clinical variables. The difference between the 2 drugs is the degree of β_1 stimulation (or blockade). Thus coupling of β_2 adrenoceptors to adenylate cyclase would appear to be enhanced with greater degrees of β_1 blockade. This implies that β_2 adrenoceptor sensitivity is inverseiy proportional to β_1 stimulation, which correlates well with the *in vitro* and *in vivo* functional data of Hall and Motomura (Hall *et al*, 1990; Hall *et al*, 1991; Motomura *et al*, 1990).

Receptor independent stimulation of adenylate cyclase by GTP was also greater in the atenolol treated group. This would suggest a functional difference at the level of the G-proteins. Changes have been noted in G-proteins in cardiac tissue from patients with end stage heart failure, with

evidence of increased levels of the inhibitory protein Gi (Bohm *et al*, 1990; Feldman *et al*, 1988; Hershberger *et al*, 1991; Neumann *et al*, 1988). This is thought to be the result of chronic stimulation by noradrenaline secondary to activation of the sympathetic nervous system. Relatively greater stimulation of β adrenoceptors in the patients treated with xamoterol when compared to atenolol might therefore be expected to have an effect on G-proteins, which could explain the greater stimulation of adenylate cyclase by GTP in the atenolol group.

Basal and forskolin stimulation of adenylate cyclase were also greater in the atenolol treated groups (table 5.4). This raises the possibility of changes in the signalling pathway at the level of the adenylate cyclase enzyme itself. Alteration in adenylate cyclase function by phosphorylation have been observed *in vitro* (Yoshimasa *et al*, 1987). In a canine model of heart failure, Marzo *et al* identified a possible defect at the level of the catalytic unit of adenylate cyclase (Marzo *et al*, 1991). Data on forskolin stimulation of adenylate cyclase should however be treated with caution since it depends on Gs for its full activity (Seamon, 1985).

Although basal and stimulated adenylate cyclase activities were lower in the xamoterol group compared to the atenolol group, they were still greater than in the small comparator group taking neither drug. If this group is truly representative of untreated patients, it has to be assumed that any change in G-proteins or catalytic unit of adenylate cyclase induced by xamoterol is in the same direction, but to a lesser extent than that of atenolol. Xamoterol was not behaving as a pure antagonist in these patients since resting heart rate was significantly greater after 5 weeks therapy with xamoterol than atenolol (table 4.3).

Although not designed to investigate the effect of calcium channel blockers on β adrenoceptor density and function, this study produces some interesting information. Receptor densities tended to be lower in patients who were concomitantly being treated with calcium channel blockers, significantly so in those treated with xamoterol (table 5.7, fig 5.4). This was associated with an insignificant trend to lower adenylate cyclase activities (table 5.8). The effect of calcium channel blockers on β adrenoceptor regulation is poorly understood. There is evidence that calcium channels and β adrenoceptors may be coregulated. Exposure of cultured chick embryo myocytes to isoprenaline reduced the density of both β adrenoceptors and calcium channels (Marsh, 1989). The reduction in calcium channels could also be produced by a increasing cAMP concentrations with forskolin and the phosphodiesterase inhibitor IBMX. In this *in vitro* model the calcium channel blocker diltiazem did not affect calcium channel density but its effect on β adrenoceptors was not investigated. In cultured neonatal rat myocytes, exposure to verapamil, diltiazem or nicardipine increased both calcium channel and β adrenoceptor density (Yonemochi *et al*, 1990). This was not prevented by lowering extracellular calcium suggesting that the effect was not mediated by lowering intracelilular calcium, although this was not directly measured.

Ferry and Kauman (1987) found a significant correlation between β adrenoceptor and calcium channel density in ventricular tissue from patients with hypertrophic obstructive cardiomyopathy but not mitral valve disease. Hedberg found that therapy with calcium channel blockers either alone or in combination with β blockers was associated with an increased density of β adrenoceptors in human right atrium (Hedberg *et al*, 1985). However Brodde *et al* (1992) found that calcium channel blockers alone had no effect on human right atrial β adrenoceptor density whereas the combination of calcium

channel blockers and β adrenoceptor antagonists was associated with upregulation of β adrenoceptors. Furthermore concomitant therapy with calcium channel blockers did not affect the downregulation of β adrenoceptors seen in patients suffering from mitral valve disease. Similarly, Jones *et al* (1990) found in a small group of patients that calcium antagonists alone did not alter human right atrial β adrenoceptor density nor the upregulation seen with β blockers. Thus both the *in vitro* and in *vivo* evidence are contradictory.

Whilst there is a potential mechanism whereby stimulation of β adrenoceptors may affect calcium channel density or function (phosphorylation by protein kinase A) there is as yet no proposed mechanism whereby calcium channel blockers could regulate β adrenoceptors. There is a potential indirect mechanism *in vivo* whereby this might occur. Therapy with several calcium channel blockers is associated with increased sympathetic nervous activity. This could in turn affect β adrenoceptor density. However this would be expected to downregulate and possibly desensitise β adrenoceptors, neither of which have previously been observed.

In conclusion this study has demonstrated that chronic therapy with the β_1 selective partial agonist xamoterol does not downregulate human cardiac β adrenoceptors. Xamoterol does not sensitise adenylate cyclase responses to the same extent as atenolol but may still enhance sensitivity relative to no drug therapy.

CHAPTER 6

Animal Study

6.1 Introduction

There are several limitations to the human study described in the 2 preceding chapters. Firstly, ethical constraints prevented the inclusion of a prospectively randomised control group on no medication. Secondly, it was impossible to selectively demonstrate β_1 adrenoceptor coupling to adenylate cyclase in human cardiac tissue. Finally, right atrial tissue was used and changes in receptors in this chamber may not entirely mirror changes in the ventricle.

Both β_1 and β_2 adrenoceptors coexist in the rat heart (Vago *et al*, 1984). Although inotropic responses appear to be mediated by the β_1 subtype (Juberg *et al*, 1985), both subtypes are coupled to adenylate cyclase (see chapter 3). Agonist induced desensitisation of receptor mediated stimulation of both adenylate cyclase activity and inotropic responses can be demonstrated *in vivo* in rat ventricle (Tse *et al*, 1979).

Rat heart therefore provides a complimentary model to the human study. In particular ventricular tissue containing β_1 adrenoceptors coupled to adenylate cyclase and inotropic responses can easily be obtained and comparison may be made to both control animals and those treated with a full agonist. Furthermore, whilst there is data on subtype differences in the time course of agonist induced changes in receptor density in this model (Lu and Barnett, 1990), there is no information on subtype differences in desensitisation of adenylate cyclase stimulation.

Previous studies have shown that whilst xamoterol may downregulate β adrenoceptors in isolated rat myocytes *in vitro* (Limas and Limas, 1990), it

does not do so in rat ventricle *in vivo* (Kowalski *et al*, 1990). This study extends the earlier *in vivo* study to examine the effect of xamoterol on the coupling of β adrenoceptor subtypes to adenylate cyclase.

6.1.1 Aim of study

The aim of this study was to determine the effect of chronic therapy with the β_1 adrenoceptor partial agonist xamoterol on the *in vivo* desensitisation of receptor mediated stimulation of adenylate cyclase activity in comparison to both control animals and those treated with the full non-selective agonist isoprenaline.

6.2 Method

Male AS rats (approximately 250 gm in weight) were infused by subcutaneous osmotic minipumps (Alza, California, USA) with either xamoterol 400 µg/kg/hr (n=6) or isoprenaline 40 µg/kg/hr (n=6). The pumps contain 200 µl and are designed to release drug at the rate of 1 µl/hr. Both drugs were made up in acidified 0.9% saline. Isoprenaline was made up to a final concentration of 10 mg/ml (hence releasing 10 µg/hr, equivalent to 40 µg/kg/hr). Xamoterol fumarate was poorly soluble and could only be made up to a final concentration of 50 mg/ml at room temperature. This required 2 pumps per animal to release a total of 100 µg/hr, equivalent to 400 µg/kg/hour. The dose of xamoterol is similar to that used in a previous study (Kowalski *et al*, 1990) in which plasma levels were achieved that were greater than those required to produce maximal increases in heart rate *in vivo*. In that study the ED_{50} of xamoterol at increasing heart rate *in vivo* was 0.36 µg/kg, which was approximately one thousand times lower than the hourly dose of xamoterol given in this experiment. The dose of isoprenaline was the same as that used in the previous study in which it produced significant downregulation of cardiac β adrenoceptors. 6 sham operated rats served as controls.

The pumps were inserted into a subcutaneous pouch fashioned at the nape of the animal's neck under general anaesthesia with halothane. After 6 days the rats were sacrificed by exsanguination under general anaesthesia and the hearts carefully removed. The great vessels, atria and right ventricle were removed and the left ventricle weighed prior to preparation of membranes as described in chapter 2.

6.2.1 Receptor binding assays

Total receptor density in specimens was determined by Scatchard analysis of saturation binding experiments using 6 different concentrations (performed in duplicate) of [^{125}I]iodopindolol ranging from 6 to 250 pM as described in chapter 2. Relative proportions of subtypes were determined by competition experiments using 18 different concentrations of CGP 20712A (performed in triplicate) and a fixed concentration of [^{125}I]iodopindolol of approximately twice its affinity (Kd) for β adrenoceptors.

6.2.2 Adenylate cyclase assays

Adenylate cyclase activity was determined in triplicate as described in chapter 3 under basal conditions (in the presence of 0.1 mM GTP), and in response to maximal stimulation by both the non-selective agonist isoprenaline (10 μM) and the β_2 partial agonist procaterol (10 μM). Non-receptor mediated stimulation was assessed in the presence of 20 μM forskolin. The relative contributions of the receptor subtypes to the stimulation of adenylate cyclase in each specimen were determined by inhibiting the stimulation of 1 μM isoprenaline with 12 different concentrations of CGP 20712A as described in chapter 3. The stimulation of adenylate cyclase by an individual subtype was calculated from the product of its relative contribution (as determined by the inhibition experiment) and the net maximal stimulation by isoprenaline (defined as that produced by 10 μM isoprenaline less basal activity).

Protein concentration was determined by the method of Lowry (Lowry *et al*, 1951) using bovine serum albumin as standards.

6.2.3 Statistics

Data are presented as means with standard deviations. Comparison of data between groups was made using the Mann Whitney test. A non-parametric test was chosen in view of the small numbers. A p value of less than 0.05 was taken as significant.

6.3 Results

Data are presented in the form of summary tables. Individual animal data are included in appendix 3.

6.3.1 Effect of drugs on left ventricular hypertrophy

Chronic therapy with β adrenoceptor agonists may cause cardiac hypertrophy (Tse *et al*, 1979). This would increase sarcolemnal surface area and hence membrane protein content. Since these assays are standardised to membrane protein, left ventricular hypertrophy would lead to a reduction in receptors per unit membrane protein without any real change in total number of receptors per cell. To estimate the size of this possible artefact, the degree of hypertrophy was determined in the 2 treatment groups by comparing left ventricular weights (as a percentage of total body weight) with those of the sham operated group (table 6.1). Isoprenaline therapy increased relative left ventricular weight by 11% whereas xamoterol had no significant effect. These results should, however, be interpreted with caution as cellular hypertrophy may occur with little change in tissue weight if cell numbers are reduced [Li et al, 1993].

Table 6.1 Left ventricular weight expressed as a percentage of total body weight by treatment group. Values are means (sd). [a]$p<0.01$ vs sham operated.

	isoprenaline (n=6)	**xamoterol** (n=6)	**sham** (n=6)
LV weight (% of body weight)	0.279 (0.008)[a]	0.261 (0.017)	0.251 (0.013)

6.3.2 Effect of drugs on receptor density

Chronic infusion with the non-selective agonist isoprenaline downregulated both β adrenoceptor subtypes, with the β_2 subtype affected relatively more (63%) than the β_1 subtype (31%) (table 6.1). Thus the proportion of β_1 adrenoceptors was significantly greater in the isoprenaline treated animals (82.5%) than in either the sham operated (71.0%) or xamoterol treated animals (72.9%). Chronic infusion with the β_1 selective partial agonist xamoterol did not significantly affect total or subtype β adrenoceptor density.

The affinity of ventricular β adrenoceptors for [^{125}I]iodopindolol was not significantly different in the 3 groups suggesting that it was unlikely that there was retained drug in the preparation.

Table 6.1 Total and subtype β adrenoceptor density and affinity constant for [^{125}I]iodopindolol binding in rat left ventricles according to treatment. % β_1 refers to proportion of total receptors that are of the β_1 subtype. Values are means (sd). $^{a}p<0.01$ vs sham, $^{b}p<0.01$ vs xamoterol

	isoprenaline (n=6)	**xamoterol** (n=6)	**sham** (n=6)
Bmax (fmols/mg)	14.93 ^{a,b} (1.41)	22.23 (2.95)	24.89 (1.59)
Kd (pM)	45 (6)	40 (12)	41 (10)
% β_1	82.5^{a,b} (3.2)	72.9 (1.3)	71.0 (2.4)
β_1 **Bmax** (fmols/mg)	12.29^{a,b} (0.97)	16.20 (2.19)	17.69 (1.43)
β_2 **Bmax** (fmols/mg)	2.64^{a,b} (0.62)	6.03 (0.83)	7.20 (0.58)

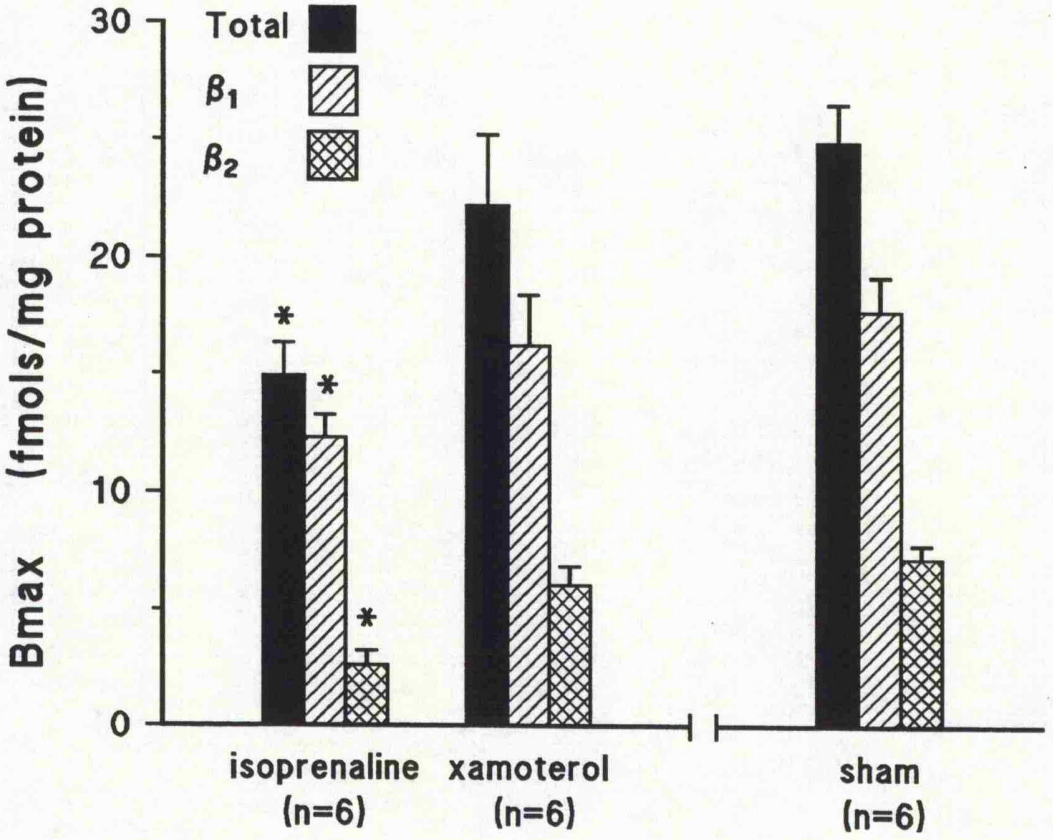


Fig 6.1 Bar chart of total and subtype β adrenoceptor density by treatment groups. Values are means with error bars of 1 standard deviation. *p<0.01 vs sham and xamoterol.

6.3.3 Effect of drugs on adenylate cyclase activity

Both isoprenaline and xamoterol infusion reduced the response to maximal stimulation of adenylate cyclase by 10 μM isoprenaline in left ventricular membranes, but the full agonist had a significantly greater effect (51%) than the partial agonist (16%) (table 6.2). Only isoprenaline infusion significantly reduced the response to stimulation of adenylate cyclase by the β_2 adrenoceptor partial agonist procaterol suggesting that chronic therapy with the β_1 selective agonist xamoterol did not affect β_2 coupling. Basal and forskolin stimulated adenylate cyclase activity were similar in all 3 groups.

Table 6.2 Basal and stimulated adenylate cyclase activity in rat left ventricle expressed as pmoles cAMP/mg protein/min. Values are means (sd). [a]p=0.005 vs sham, [b]p=0.02 vs sham, [c]p=0.005 vs xamoterol

	isoprenaline (n=6)	**xamoterol** (n=6)	**sham** (n=6)
basal	6.89 (1.03)	5.73 (1.38)	5.32 (1.20)
isoprenaline (10 μM)	21.94 (2.67)[a,c]	37.41 (3.95)[b]	44.57 (3.85)
procaterol (10 μM)	10.71 (1.57)[a]	15.33 (2.07)	17.46 (2.43)
forskolin (20 μM)	85.06 (13.86)	99.37 (12.42)	101.17 (13.87)

Analysis of the subtype contributions to isoprenaline stimulation revealed that the desensitisation induced by chronic infusion with the β_1-selective agonist xamoterol was limited to the β_1 subtype whereas the non-selective agonist isoprenaline desensitised both subtypes (table 6.3, fig 6.2). The reduction in net β_1 stimulation by isoprenaline was less in the animals treated with the full agonist (60%) than in those treated with the partial agonist (28%).

Table 6.3 Contribution of individual subtypes to net stimulation (total less basal) of adenylate cyclase by isoprenaline. Adenylate cyclase activity expressed as pmoles cAMP/mg protein/min. Values are means (sd). [a]p=0.005 vs sham, [b]p=0.008 vs sham, [c]p=0.013 vs sham, [d]p=0.005 vs isoprenaline, [e]p=0.005 vs xamoterol

	isoprenaline (n=6)	**xamoterol** (n=6)	**sham** (n=6)
net isoprenaline (10 μM)	15.05 (2.02)[a,e]	31.68 (2.96)[b]	39.25 (3.00)
% β1	69.7 (2.4)	59.9 (2.2)[c,d]	67.2 (4.2)
net β_1 stimulation	10.50 (1.52)[a,e]	18.94 (1.24)[a]	26.43 (3.38)
net β_2 stimulation	4.56 (0.63)[a,e]	12.74 (1.78)	12.82 (1.16)

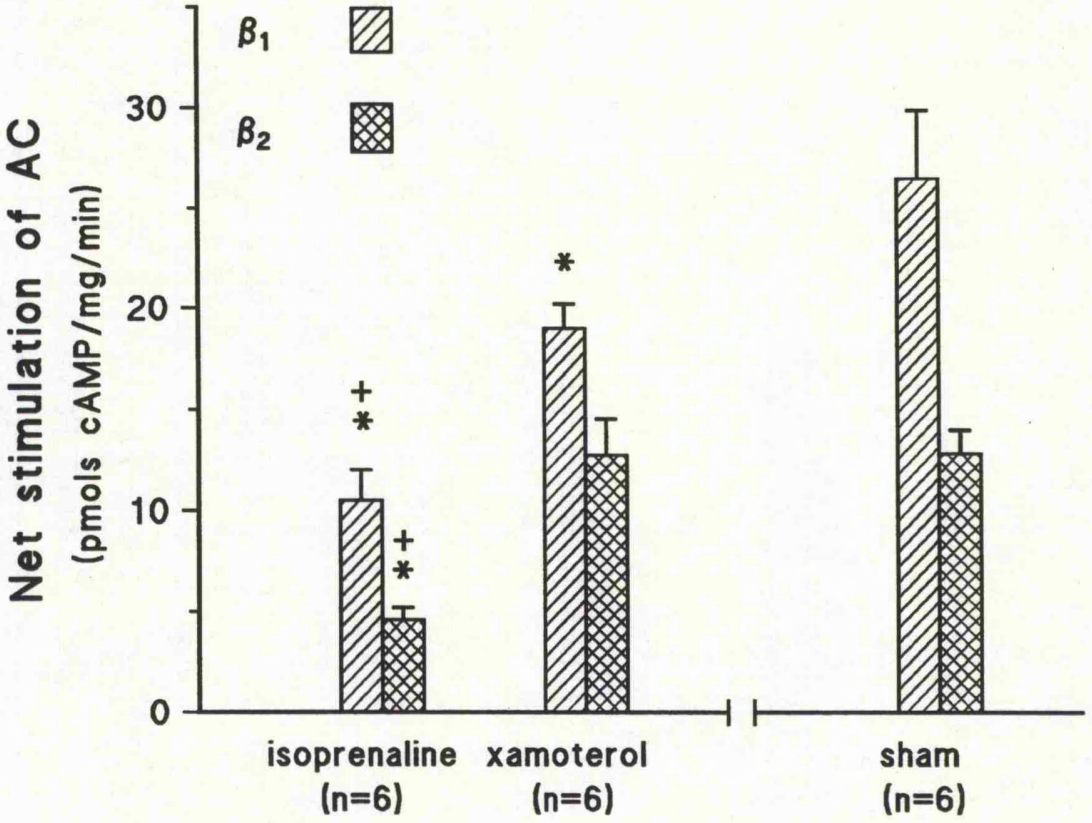


Fig 6.2 Bar chart of β adrenoceptor subtype contribution to the stimulation of adenylate cyclase by 10 μM isoprenaline. Data given as means with error bars of 1 standard deviation. *p=0.005 vs sham, $^{+}$p=0.005 vs xamoterol

6.4 Conclusions

The main aim of this study was to examine the effects of chronic xamoterol therapy on the coupling of ventricular β_1 adrenoceptors to adenylate cyclase. The animal model was chosen because, unlike the human model, β_1 adrenoceptor coupling could be easily demonstrated. The results have shown that the β_1 adrenoceptor partial agonist xamoterol selectively desensitises β_1 adrenoceptor mediated stimulation of adenylate cyclase but to a considerably lesser degree than the full agonist isoprenaline which desensitises both subtypes.

Desensitisation may occur as a result of a reduction in receptor numbers, a reduction in the coupling of the receptors to adenylate cyclase or a combination of both. The binding studies confirmed the results of a previous study (Kowalski *et al*, 1990) showing that xamoterol did not alter receptor density. Thus the desensitisation induced by xamoterol infusion must have been mediated by an effect on the coupling of receptors to adenylate cyclase. The site of this action is not clear. Unlike the human study, basal (in the presence of GTP) and forskolin stimulation of adenylate cyclase were similar in all three treatment groups suggesting no significant change in G-protein or adenylate cyclase function. Receptor affinity for antagonists was unchanged as demonstrated by the affinity constants for binding of [^{125}I]iodopindolol. It has previously been demonstrated that neither isoprenaline or xamoterol treatment affects the affinity of receptors for agonists (Kowalski *et al*, 1990). Thus alterations in receptor affinity are unlikely to explain the differences. A possible mechanism would be an alteration of receptor binding to G proteins. [^{125}I]iodopindolol is a relatively lipophilic ligand and will label all β

adrenoceptors even those translocated within the membrane to a site unavailable to G-proteins.

Isoprenaline infusion downregulated both subtypes and thus it is impossible to determine whether the observed desensitisation was the result of changes in receptor density alone or whether there was some uncoupling of receptors from adenylate cyclase as well. The EC_{50} for isoprenaline stimulation of adenylate cyclase as shown in chapter 3 was 65 nM. The affinity of isoprenaline for rat cardiac β adrenoceptors as determined from isoprenaline displacement of [^{125}I]iodopindolol binding was 20 nM (mean of 3 experiments, data not shown). Since these 2 figures are similar, it is unlikely that there are significant numbers of spare receptors in the stimulation of adenylate cyclase. Thus receptor mediated stimulation of adenylate cyclase activity would be very sensitive to the changes in receptor density caused by the isoprenaline infusion. It is interesting to note that Kowalski *et al* (1990) found the EC_{50} for isoprenaline mediated chronotropic responses in isolated rat atria to be considerably lower (0.45 nM) than that for stimulation of adenylate cyclase observed here. This would suggest that the difference between receptor occupancy and chronotropic response (ie "spare" receptors) occurs at a level after the generation of the second messenger cAMP.

An interesting observation from this study was the fact that β_2 adrenoceptors were downregulated to a greater extent by isoprenaline than β_1 adrenoceptors. This has been observed in previous studies (Kowalski *et al*, 1990; Lu and Barnett, 1990; Thomson *et al*, 1992). Using autoradiographic techniques, Molenaar *et al* (1990) demonstrated greater downregulation of β_2 adrenoceptors in all areas of the guinea pig heart in response to isoprenaline infusion. It would appear therefore that rodent cardiac β_2 adrenoceptors are more susceptible to downregulation by isoprenaline than are β_1

adrenoceptors. The mechanism for this difference is not clear. It is possible that because neuronally released noradrenaline is a relatively β_1 selective agonist, β_1 adrenoceptors are under greater tonic downregulation than the β_2 subtype and hence less responsive to further downregulation. An alternative would be that β_2 adrenoceptors are sited on cells with greater exposure to circulating isoprenaline such as endothelial cells.

This study was designed to complement the human study with particular reference to the desensitisation of β_1 adrenoceptors. It has shown that chronic therapy with the β_1 adrenoceptor partial agonist xamoterol can desensitise β_1 mediated stimulation of adenylate cyclase in cardiac tissue, but does so considerably less than the full agonist isoprenaline. Xamoterol has no effect on β_2 mediated stimulation of adenylate cyclase unlike the non selective agonist isoprenaline. Thus this study demonstrates that in the rat heart, desensitisation can occur in a subtype selective fashion and that the degree to which desensitisation occurs is related to the intrinsic activity of the agonist producing it.

CHAPTER 7

Conclusions

At the time this study was conceived (1989) evidence was accumulating to suggest that β adrenoceptor regulation has an important role in the pathophysiology and therapy of heart failure. It was known that cardiac β adrenoceptors were downregulated and uncoupled from adenylate cyclase in heart failure with consequent tissue subsensitivity to catecholamine stimulation. Chronic therapy with β adrenoceptor agonists was of limited use because of the rapid development of tolerance presumed to be the result of receptor desensitisation. Chronic therapy with β adrenoceptor antagonists had been shown in several uncontrolled clinical trials to produce beneficial effects in selected patients with heart failure and this was associated with increases in β adrenoceptor density.

On this background, the introduction of the partial agonist xamoterol represented a novel approach to the treatment of heart failure. Theoretically it would be a weak agonist at rest and yet limit excessive sympathetic stimulation on exercise, hence "modulating" the sympathetic nervous stimulation of the heart. In clinical trials xamoterol had shown considerable promise in the treatment of mild to moderate heart failure and in particular there was no evidence of tolerance to its effects over periods of up to 18 months. This would suggest that, in contrast to a full agonist, xamoterol did not desensitise human cardiac β adrenoceptors. It was a logical step therefore to study the effect of this drug on the regulation of human cardiac β adrenoceptors.

The model chosen for this study was patients undergoing CABG from whom myocardial tissue could be easily obtained. Like all human studies, this had limitations, the most significant being the ethical constraints at including a prospectively randomised placebo control group. Prospectively randomised comparisons were made between xamoterol therapy and treatment with a

drug (atenolol) whose effect on β adrenoceptors was already described. The fact that xamoterol produced identical changes in receptor densities to those of atenolol strongly supports the hypothesis that xamoterol does not downregulate human cardiac β adrenoceptor *in vivo*. This may be particularly relevant in human cardiac tissue where there are few spare receptors and maximal functional response is dependent on receptor density.

The data on the coupling of receptors to adenylate cyclase is more difficult to interpret since adenylate cyclase activities were significantly higher in the atenolol group than in the xamoterol group. From this it can be safely concluded that the 2 drugs have different effects but it is not clear whether xamoterol alters coupling relative to treatment without β-blockers. An indication can be gained from the small comparator group of patients not taking β-blockers at the time of surgery in whom adenylate cyclase activities were markedly lower than in the xamoterol group. Direct statistical comparison between this small non-randomised group of patients has been avoided in this study, but it is reasonable to surmise from the data in this group that adenylate cyclase activity was not reduced by xamoterol therapy compared to therapy without β-blockers.

Thus chronic xamoterol therapy does not downregulate human cardiac β adrenoceptors and is unlikely to desensitise receptor mediated stimulation of adenylate cyclase. The clinical implication of this is that tolerance is unlikely to develop to the agonist effects of xamoterol. This is consistent with the observed clinical data. The large efficacy trials were able to demonstrate a clinical benefit after 3 months treatment (The German and Austrian Xamoterol Study Group, 1988; Waller *et al*, 1989) and a smaller study found no evidence of tolerance after 18 months therapy (Vigholt-Sorenson and Faergeman, 1990).

The human study produces an incomplete picture of the pharmacological effects of xamoterol on the regulation of β adrenoceptor coupling to adenylate cyclase for 3 reasons. Firstly it was only possible to reliably demonstrate coupling of β_2 adrenoceptors to adenylate cyclase. Secondly it was impossible to study the effects of a full agonist. Finally there was no placebo control group. In order to extend the human data therefore, an animal model was used. The rat heart was a particularly suitable model to complement the human study since β_1 coupling to adenylate cyclase could be easily demonstrated and stimulation of cardiac β_1 receptors produce physiological responses.

As in the human study, xamoterol therapy was not associated with receptor downregulation in the rat heart. β_2 adrenoceptor coupling to adenylate cyclase was unaffected by xamoterol therapy showing that in this model also, chronic therapy with xamoterol does not desensitise β_2 adrenoceptor mediated response. There was however selective desensitisation of β_1 adrenoceptor mediated stimulation of adenylate cyclase, which in the absence of changes in receptor densities must represent uncoupling of β_1 receptors from adenylate cyclase. Agonist induced desensitisation appears to be a graded response, depending on the strength of the agonist used. Hence chronic infusion with the full agonist isoprenaline reduced β_1 stimulation of adenylate cyclase by 60% whereas infusion with the partial agonist xamoterol reduced it by only 28%. Thus coupling of rat cardiac β adrenoceptors can be modified in a subtype selective fashion and the extent to which an agonist desensitises receptors is related to its intrinsic activity.

It is possible that chronic xamoterol therapy does desensitise human cardiac β_1 adrenoceptors in a similar manner. However even if this was the case, it

would be likely that any desensitisation would be considerably less than that following therapy with an agonist with greater intrinsic activity (such as dobutamine). It should be born in mind that xamoterol is a relatively weak agonist in human myocardium, requiring the presence of forskolin or a phosphodiesterase inhibitor to demonstrate an inotropic effect *in vitro* (Bohm *et al*, 1990).

Caution should be exercised in extrapolating the results of the animal study to man, since there are considerable differences between the rat heart and that of the human. Physiological effects appear to be almost entirely mediated by the β_1 subtype in the rat (Juberg *et al*, 1985). It is possible to culture cells from neonatal rats which are rich in either β_1 or β_2 adrenoceptors suggesting that the 2 receptor subtypes exist on different cells (Lau *et al*, 1980). In the human heart both subtypes mediate physiological responses and recent evidence suggest that they may coexist on the same cell (del Monte *et al*, 1993). The potential for cross regulation of subtype signalling pathways is obviously greater in the human heart. There are spare receptors in the rat heart but not in the human heart (Brown *et al*, 1992). This means that the human heart will be more sensitive to changes in receptor density than the rat heart. And finally xamoterol is a weaker agonist in human than rat heart and it is quite conceivable that it would produce different effects in the 2 species.

Although this project was conceived to investigate regulation of cardiac β adrenoceptors by xamoterol, several interesting clinical and pharmacological observations have been made in addition. On the clinical side, in these selected patients, xamoterol was shown to be an effective anti-anginal agent as judged by symptoms. It was less likely to cause profound bradycardias during surgery and reduced the requirement for inotropic support post-

operatively. It significantly reduced diuretic requirements in the early post-operative phase. This benefit did not appear to be at the cost of an excess of significant rhythm disturbances. This raises the possibility that xamoterol may be a useful alternative to atenolol in the peri-operative period in patients undergoing CABG, perhaps especially in patients with impaired left ventricular function.

On the pharmacological side, the evidence that β_2 adrenoceptor coupling was inversely related to β_1 stimulation in the human heart supports earlier data suggesting that β_1 selective antagonists sensitise β_2 mediated responses. The data on the effect of concomitant therapy with calcium antagonists suggest an interaction between β adrenoceptor agonists and calcium antagonists not previously noted (and as yet unexplained). In the rat model, this was the first demonstration of subtype selective regulation of coupling *in vivo* in this species.

As mentioned above, xamoterol was showing great promise in the treatment of heart failure at the time this study was conceived. However a study published in 1990 curtailed further development of the drug for this indication (The xamoterol in severe heart failure group, 1990). 516 patients with NYHA class III and IV heart failure despite treatment with diuretics and angiotensin converting enzyme inhibitors were randomised to receive in addition either xamoterol (n=352) or placebo (n=164). Mortality was significantly greater in the xamoterol group (9.1%) than the placebo group (3.7%) at 100 days if analysed on an intention to treat basis. The increase in deaths was equally divided between progression of heart failure and sudden death. The study was designed to look at efficacy and not mortality and it is not possible to determine the mechanisms for the increase in mortality. However it would appear, as might be predicted from its pharmacological properties, that

xamoterol had considerable antagonist properties in these patients with severe heart failure. 24 hour holter monitoring in a subgroup of patients showed that daytime heart rate was reduced by xamoterol (ie an antagonist effect). On entry, mean plasma noradrenaline levels were 776 pg/ml in the placebo group and 678 pg/ml in the xamoterol group, both levels above that at which Sato had found xamoterol to act as a β adrenoceptor antagonist (Sato *et al*, 1987). Although nocturnal heart rate was increased by xamoterol suggesting an agonist effect by night, there was no evidence of a pro-arrhythmic effect in those patients who had 24 hour monitoring.

Other studies have shown a beneficial effect of β-blocker therapy in selected patients with heart failure. However these used carefully titrated doses of drugs, unlike this study which used a maximal dose of xamoterol (200 mg bd) from the start. It should also be noted that β-blockers are most effective in idiopathic dilated cardiomyopathy, whereas in the xamoterol study the majority of patients had ischaemic heart disease.

The fact that xamoterol therapy is associated with increased mortality in patients with severe heart failure has important clinical implications. Since the natural history of heart failure is for it to progress, if xamoterol does not influence this progression, patients prescribed xamoterol when in the early stages of the disease would end up taking it when they had severe heart failure. Because of this study, the indications for use of xamoterol were severely curtailed and further development of the drug was halted. It is fruitless to speculate on the outcome had the severe heart failure study not been conducted or had it been designed differently (titration of dose, selection of patients).

The partial agonist properties of xamoterol remain of interest and there are other potential clinical indications where this might be useful. Xamoterol helps to control the ventricular response to atrial fibrillation either alone or in combination with digoxin. In particular, the partial agonist property helps prevent ventricular pauses when sympathetic tone is low whilst limiting heart rate on exercise, although there is no evidence that this translates into symptomatic improvements (Ang *et al*, 1990; Lewis *et al*, 1989; Lundstrom *et al*, 1992). In patients with impaired left ventricular function and ventricular tachycardia inadequately controlled with amiodarone, xamoterol is as effective as metoprolol as adjunctive therapy with the added benefit of improving exercise tolerance (Bashir *et al*, 1992). In the Shy-Drager syndrome xamoterol may reduce the incidence of postural hypotension (Obara *et al*, 1992). It has been proposed that xamoterol may be of use in patients with hypertrophic cardiomyopathy by enhancing diastolic function and reducing exercise induced tachycardia although Gilligan *et al* (1992) failed to find evidence of the former effect. In all these applications which involve the "modulation" of sympathetic tone, the data contained within this thesis has clinical relevance.

This thesis would be complimented by a functional assessment of the consequences of the observed biochemical changes. This would be particularly important in view of the discrepancy between subtype coupling to adenylate cyclase and functional responses in man. This could be performed *in vitro*, using atrial muscle strips from patients udergoing surgery randomised pre-operatively as in this study, and *in vivo* in chronically treated normal subjects (after an adequate washout) using heart rate response to exercise and salbutamol infusion to assess β_1 and β_2 mediated effects respectively.

Unless a major new indication for xamoterol therapy is found, further work on its effect on β adrenoceptor regulation is unlikely to be pursued for clinical reasons. It does however remain a useful tool for investigating mechanisms of receptor regulation, particularly as it can be given to patients in circumstances where a full agonist would be contra-indicated. This enabled a comparison of 2 drugs with differing intrinsic activities at the β_1 subtype to be studied in a prospectively randomised fashion in the current study.

Although enhancement of β_2 mediated effects by β_1 blockade has previously been demonstrated, the mechanism remains unknown. An inverse relationship between the stimulation of β_1 adrenoceptors and β_2 mediated activation of adenylate cyclase was well demonstrated in the human study presented in this thesis. A unique finding of this work was the demonstration that this sensitisation may occur at the level of the adenylate cyclase enzyme. Whereas changes in G-proteins have been extensively studied, regulation of the signalling pathway at the level of adenylate cyclase has not. Marzo *et al* (1991) demonstrated a possible defect at this level In a canine model of heart failure induced by chronic ventricular pacing. Yoshimasa *et al* (1987) demonstrated phosphorylation of adenylate cyclase in rat erythrocytes by stimulating protein kinase C with a phorbol ester resulting in enhanced adenylate cyclase activity. It is interesting to note that Motomura *et al* also found an enhanced response to forskolin following chronic therapy with β_1 adrenoceptor antagonists (Motomura *et al*, 1990).

One of the problems in studying this further is the fact that no assay exists for directly quantifying the adenylate cyclase enzyme. However functional activity could be assessed using the technique that Marzo *et al* used in their dog model. This involved the stimulation of adenylate cyclase in a membrane preparation by adding Gs purified from rabbit liver and activated with GTP-γ-

S. In addition, the response to stimulation of other receptors linked to adenylate cyclase, such as the histamine receptor, could be determined. It would be expected that these would also be enhanced if there had been a change at the level of adenylate cyclase. Further studies could also be performed on changes in G-proteins using ADP ribosylation, immunoblotting and reconstitution into cyc- cell lines to form a more precise picture of the changes in the signalling pathway.

APPENDIX 1

Clinical results

Patient details

Xamoterol group

trial no	sex	age	vessels	ischaemia score	LVEF - angio	LVEF - MUGA
2	m	55	3	12.2	57	51
4	m	51	3	12.3	55	60
5	m	65	3	11.6	62	62
8	m	56	3	12.2	53	52
10	m	52	3	13.7	40	52
12	m	42	3	12.4	75	64
14	m	54	3	5.8	62	59
16	f	61	3	11.1	74	58
17	m	59	3	10.8	45	56
20	m	56	3	11.5	38	42
22	m	54	3	9.5	65	57
24	f	50	3	12	71	69
25	m	58	3	12.8	41	51
28	m	50	2	8.3	61	63
30	m	56	3	12.8	65	53
31	m	63	3	11.2	67	*
33	m	44	2	10.4	60	68
35	m	66	3	13.5	50	59
37	f	59	2	11.7	46	*
40	m	54	2	8.4	60	64
41	m	55	2	11.3	64	67
44	m	66	3	8.9	56	*
45	m	65	2	9.5	64	59
51	m	63	3	11.2	67	61
54	m	64	3	10.7	63	*
55	m	59	3	12.5	50	58
57	m	64	3	12.7	37	36
60	m	62	3	12.8	58	65

"vessels" = number of diseased (>50% diameter stenosis) coronary arteries
"LVEF" = left ventricular ejection fraction

Atenolol group

trial no	sex	age	vessels	ischaemia score	LVEF - angio	LVEF - MUGA
1	m	49	3	10.0	65	57
3	m	48	3	13.1	67	*
6	m	47	3	11.3	46	62
7	m	61	3	8.5	61	66
9	m	49	3	11.7	54	53
11	m	58	3	13.2	53	69
13	m	50	2	10.3	61	56
15	m	60	3	13.4	57	49
18	m	52	2	9.3	67	52
19	m	69	3	11.3	65	64
21	m	43	3	12.7	70	49
23	m	61	2	9.8	66	63
26	m	51	3	10.3	70	56
27	m	49	3	10.4	60	66
29	f	39	2	9.3	66	69
32	f	54	3	12.6	58	69
34	m	58	2	10.3	70	50
36	m	66	3	10.2	70	62
38	m	60	3	11.2	62	58
39	m	48	3	11.2	71	*
42	m	51	3	14.1	48	58
43	m	64	2	10.1	62	58
52	m	54	3	11.3	70	58
53	m	31	3	13.5	34	*
56	m	59	3	12.4	56	54
58	m	54	3	13.0	65	48
59	m	46	3	10.6	72	65

Concomitant therapy

Xamoterol group

trial no	β-blocker	calcium antagonist	nitrate
2	atenolol	none	ISMN
4	atenolol	none	ISMN
5	none	diltiazem	ISMN
8	atenolol	nifedipine	ISMN
10	metoprolol	none	none
12	none	none	none
14	atenolol	nifedipine	ISMN
16	none	diltiazem	ISMN
17	atenolol	nifedipine	GTN patch
20	atenolol	diltiazem	ISMN
22	atenolol	none	ISMN
24	atenolol	none	none
25	atenolol	nifedipine	ISMN
28	atenolol	none	none
30	atenolol	none	none
31	atenolol	none	none
33	none	diltiazem	none
35	atenolol	alodipine	ISMN
37	atenolol	nifedipine	none
40	bisoprolol	nifedipine	none
41	atenolol	none	none
44	atenolol	nifedipine	ISMN
45	metoprolol	diltiazem	ISMN
51	atenolol	none	none
54	atenolol	none	none
55	propanolol	nifedipine	none
57	propanolol	nifedipine	ISMN
60	metoprolol	diltiazem	ISMN

"ISMN" = isosorbide mononitrate

Atenolol group

trial no	β-blocker	calcium antagonist	nitrate
1	atenolol	none	none
3	atenolol	diltiazem	none
6	atenolol	nifedipine	none
7	atenolol	nifedipine	ISMN
9	none	diltiazem	none
11	atenolol	none	none
13	atenolol	none	ISMN
15	atenolol	nifedipine	ISMN
18	metoprolol	none	none
19	atenolol	nifedipine	ISMN
21	metoprolol	diltiazem	none
23	atenolol	nifedipine	ISMN
26	none	nicardipine	ISMN
27	atenolol	nifedipine	none
29	atenolol	diltiazem	ISMN
32	atenolol	none	ISMN
34	atenolol	nifedipine	none
36	atenolol	nicardipine	none
38	atenolol	none	none
39	atenolol	none	none
42	none	diltiazem	ISMN
43	atenolol	nifedipine	ISMN
52	atenolol	diltiazem	ISMN
53	atenolol	none	none
56	acebutalol	nifedipine	none
58	atenolol	diltiazem	none
59	atenolol	none	ISMN

Resting pulse and blood pressure

Xamoterol group

trial no	on entry				pre-op			
	pulse	systolic	diastolic	RPP	pulse	systolic	diastolic	RPP
2	60	150	80	9000	*	*	*	*
4	68	140	80	9520	84	135	100	11340
5	66	170	90	11220	*	*	*	*
8	64	130	65	8320	82	130	66	10660
10	62	150	80	9300	60	124	76	7440
12	84	140	80	11760	70	126	70	8820
14	58	130	74	7540	72	140	94	10080
16	58	144	76	8352	60	106	68	6360
17	58	140	82	8120	75	150	108	11250
20	54	142	76	7668	*	*	*	*
22	52	128	70	6656	78	132	94	10296
24	72	122	74	8784	*	*	*	*
25	60	102	62	6120	82	126	84	10332
28	54	118	66	6372	*	*	*	*
30	56	180	100	10080	72	176	108	12672
31	48	160	70	7680	*	*	*	*
33	60	104	60	6240	74	114	60	8436
35	42	148	62	6216	72	136	80	9792
37	64	160	90	10240	*	*	*	*
40	72	140	80	10080	70	156	80	10920
41	53	170	84	9010	78	160	86	12480
44	48	166	84	7968	*	*	*	*
45	60	154	86	9240	78	176	90	13728
51	54	156	70	8424	57	150	66	8550
54	60	166	85	9960	*	*	*	*
55	54	140	80	7560	72	156	90	11232
57	53	140	74	7420	70	132	64	9240
60	50	110	66	5500	66	128	86	8448

"RPP" = rate pressure product (product of systolic blood pressure and heart rate)

Atenolol group

trial no	on entry				pre-op			
	pulse	systolic	diastolic	RPP	pulse	systolic	diastolic	RPP
1	64	130	70	8320	60	110	80	6600
3	60	124	70	7440	*	*	*	*
6	60	144	90	8640	53	124	68	6572
7	44	160	78	7040	60	120	56	7200
9	72	160	90	11520	60	134	72	8040
11	58	168	80	9744	44	138	70	6072
13	64	152	80	9728	64	130	76	8320
15	56	142	80	7952	66	130	74	8580
18	64	118	76	7552	70	114	66	7980
19	64	154	68	9856	66	146	72	9636
21	64	108	64	6912	*	*	*	*
23	50	112	58	5600	53	152	74	8056
26	66	116	74	7656	52	98	64	5096
27	50	172	84	8600	63	140	74	8820
29	84	148	90	12432	64	126	70	8064
32	40	160	74	6400	56	150	72	8400
34	72	142	88	10224	56	138	80	7728
36	58	140	78	8120	60	140	80	8400
38	43	158	74	6794	46	168	70	7728
39	50	172	74	8600	*	*	*	*
42	65	164	76	10660	43	158	68	6794
43	59	114	68	6726	64	142	80	9088
52	48	116	70	5568	52	120	72	6240
53	56	122	58	6832	*	*	*	*
56	68	154	90	10472	62	124	82	7688
58	54	124	72	6696	49	136	86	6664
59	58	126	78	7308	50	120	78	6000

Weekly angina attack rates - xamoterol group

trial no	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
5	0	0	0	1	0	0	0	0
10	7	8	5	11	8	2		
14	31	36	36	54	56	64		
16	3	7	3	2	3	5		
17	0	3	3	3	1	3		
25	6	9	11	8	9	4		
30	3	8	2	4				
33	5	3	5	2	0	2		
35	2	4	0	0	3			
41	0	0	1	1	1	1		
45	12	14	15	16	14	17		
51	0	0	0	0	0			
55	43	44	30	25	39			
57	3	2	1	3	2	4		
60	27	14	11	8	6	5		

Weekly GTN consumption - xamoterol group

trial no	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
5	0	0	0	0	0	0	0	0
10	14	13	12	17	11	7		
14	28	35	36	54	55	63		
16	3	7	3	2	3	5		
25	6	9	11	8	9	4		
30	3	8	2	4				
33	0	0	0	0	0	0		
35	0	0	0	0	0			
41	0	0	2	2	1	1		
45	24	30	31	32	28	34		
51	*	*	*	*	*			
55	*	*	*	*	*			
57	4	3	3	5	2	5		
60	45	39	46	40	42	38		

Weekly angina attack rates - atenolol group

trial no	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
1	10	9	12	9				
11	3	4	4	3	4			
15	7	5	4	1	13			
18	2	4	4	4	3			
19	0	0						
32	16	12	10	10	6	10		
34	25	25	26	24	23			
36	10	8	17	8				
38	0	0	0	0	0			
42	0	0	0	0	0			
43	5	9	7	7	6			
56	0	0	2	0	2	0		
58	11	11	11	14	13	14		

Weekly GTN consumption - atenolol group

trial no	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
1	10	9	12	9				
11	3	4	4	3	4			
15	18	20	19	14	33			
18	0	0	0	0	0			
19	*	*						
32	*	*	*	*	*	*		
34	19	19	21	17	16			
36	13	8	17	8				
38	*	*	*	*	*			
42	0	0	0	0	0			
43	5	9	7	7	6			
56	0	0	0	0	0	0		
58	3	3	4	7	1	3		

Mean hourly heart rate - xamoterol group (pre-op)

	hour (from midnight)											
trial no	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**
4	79	86	81	81	76	75	81	82	82	87	87	83
8	93	93	96	97	94	95	*	*	*	*	*	*
10	68	75	66	73	67	68	68	73	*	*	*	*
12	63	64	61	64	64	64	68	72	74	75	71	74
14	77	77	71	75	70	71	80	79	71	73	72	*
16	62	60	59	60	58	61	63	67	66	64	63	64
17	69	71	69	75	75	68	77	82	82	78	81	78
22	76	71	71	77	78	78	85	91	87	84	85	86
30	88	87	89	88	83	84	90	91	91	88	82	83
33	86	83	85	76	74	71	71	71	73	83	*	*
35	63	66	69	68	68	69	63	68	7	80	82	73
45	67	64	63	62	64	65	72	71	72	70	70	68
51	79	82	80	83	74	72	77	76	79	71	73	71
57	78	70	68	73	69	72	74	74	77	80	76	68
60	67	71	67	65	65	60	83	76	74	70	70	66
mean	**74**	**75**	**73**	**74**	**72**	**72**	**75**	**77**	**77**	**77**	**76**	**74**
sd	10	10	11	10	9	9	8	8	7	7	7	8

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
4	86	81	80	79	74	85	82	85	83	84	83	74
8	*	*	*	*	*	91	87	84	84	85	*	89
10	*	86	76	71	70	74	67	70	69	72	68	62
12	78	78	73	72	70	76	77	75	70	68	68	66
14	*	78	77	74	76	77	78	81	80	77	80	76
16	68	70	69	68	70	70	69	66	63	60	60	61
17	83	86	79	77	76	78	83	84	79	78	76	68
22	98	89	81	84	87	89	85	87	81	81	75	74
30	91	95	91	86	79	83	88	88	86	81	80	82
33	82	92	90	81	84	95	93	92	91	88	86	85
35	70	77	77	*	69	70	74	73	70	68	67	62
45	79	82	79	79	79	86	82	77	77	77	72	64
51	75	71	67	66	66	75	68	62	66	67	67	76
57	78	80	80	78	73	76	78	77	73	66	68	73
60	71	77	79	73	73	70	70	69	67	68	70	69
mean	**80**	**82**	**78**	**76**	**75**	**80**	**79**	**78**	**76**	**75**	**73**	**72**
sd	9	7	7	6	6	8	8	9	8	8	7	9

Mean hourly heart rate - atenolol group (pre-op)

	hour (from midnight)											
trial no	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**
1	62	62	64	60	61	60	68	65	65	63	61	61
6	59	59	59	57	52	56	59	57	58	57	56	56
7	55	53	52	53	53	51	*	*	*	*	*	*
9	61	55	57	58	59	63	68	66	66	62	63	59
11	48	46	44	45	46	46	54	54	48	45	44	*
13	55	55	54	56	51	53	62	58	58	57	55	61
15	64	57	58	57	58	65	63	69	68	63	*	*
18	55	56	51	57	55	52	71	78	71	66	65	66
19	51	51	49	53	53	49	68	71	82	85	68	62
23	53	53	52	49	49	48	50	48	44	44	43	43
27	62	62	60	60	61	69	83	73	66	61	*	*
29	59	60	59	57	60	61	70	73	80	72	68	64
34	61	58	60	61	64	61	59	65	75	76	70	67
36	61	57	52	52	52	53	53	60	63	61	56	55
38	45	46	47	44	45	44	45	48	47	49	47	44
42	41	43	45	45	41	41	43	50	45	47	47	45
43	62	61	59	57	63	68	65	69	64	59	62	64
52	57	61	60	60	58	59	60	64	67	58	54	*
58	44	43	43	51	51	53	50	53	54	49	47	47
mean	**56**	**55**	**54**	**54**	**54**	**55**	**61**	**63**	**62**	**60**	**57**	**57**
sd	7	6	6	5	6	8	10	9	11	11	9	9

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	63	67	63	58	61	67	68	68	65	61	63	61
6	*	60	61	57	53	56	60	63	58	58	56	59
7	56	56	55	53	55	60	55	53	54	53	54	55
9	62	62	61	59	59	68	70	67	65	62	58	58
11	*	49	49	49	45	54	52	48	50	51	49	50
13	60	62	62	60	66	65	65	63	60	59	57	56
15	*	*	*	*	*	*	72	71	73	69	63	64
18	68	76	71	70	67	73	76	72	69	61	66	53
19	59	62	59	66	60	63	65	68	70	74	80	66
23	47	48	49	50	52	51	55	56	49	48	48	52
27	69	62	68	61	61	68	66	66	68	70	75	70
29	67	68	68	67	65	70	71	69	64	67	64	58
34	64	71	70	69	64	63	64	66	59	70	66	59
36	53	60	61	61	62	60	64	64	65	60	64	60
38	43	46	51	49	50	48	50	52	48	49	47	49
42	48	51	50	45	45	48	48	51	49	44	43	43
43	63	70	67	68	68	68	60	60	58	58	64	65
52	52	55	54	52	52	58	55	56	53	54	52	52
58	51	55	*	56	53	57	61	55	54	51	48	50
mean	**58**	**60**	**60**	**58**	**58**	**61**	**62**	**62**	**60**	**59**	**59**	**57**
sd	8	9	8	8	7	8	8	7	8	9	10	7

Mean hourly heart rate - xamoterol group (post-op)

	hour (from midnight)											
trial no	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**
4	95	91	93	93	91	88	88	93	91	98	97	95
8	93	94	96	97	97	97	98	96	97	96	96	94
10	76	77	84	82	83	90	90	88	85	87	85	84
12	86	84	84	84	87	89	91	86	89	85	87	89
14	77	79	79	83	90	88	89	76	74	75	77	*
16	72	73	72	72	73	74	75	73	71	70	*	*
17	86	88	88	87	84	88	86	90	90	*	*	*
22	97	93	89	94	95	98	97	85	94	95	95	103
25	87	88	87	87	87	89	93	100	101	98	95	94
30	90	89	89	90	93	93	92	95	98	96	93	92
33	92	91	91	89	90	91	90	86	84	90	98	*
35	71	70	73	71	72	74	75	78	73	75	76	72
40	82	85	84	84	85	82	83	83	88	89	88	*
41	89	87	80	83	88	84	86	83	87	91	*	77
45	69	73	73	74	76	83	87		80	78	84	80
51	85	82	84	88	86	87	90	84	84	*	*	*
55	73	76	77	72	80	74	72	78	77	75	74	*
60	96	94	91	88	87	89	94	94	96	89	87	85
mean	**84**	**84**	**84**	**84**	**86**	**87**	**88**	**86**	**87**	**87**	**88**	**88**
sd	9	8	7	8	7	7	7	8	9	9	8	9

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
4	*	98	94	95	97	94	97	93	95	95	97	97
8	98	96	98	99	95	99	97	97	94	92	92	94
10	91	91	91	95	87	89	93	87	88	88	88	84
12	90	86	87	88	89	90	88	85	87	89	91	87
14	79	78	80	73	80	78	83	87	83	84	82	77
16	72	69	75	75	73	73	76	74	74	75	75	70
17	*	*	*	89	86	85	86	88	87	88	90	86
22	106	107	104	102	102	103	103	102	99	103	99	97
25	99	96	91	92	95	98	100	106	103	102	97	85
30	92	96	97	95	97	98	100	100	95	95	95	97
33	94	106	100	90	91	92	95	102	100	96	93	95
35	*	79	77	75	74	73	75	77	78	77	76	72
40	88	87	89	85	81	80	80	83	83	85	85	84
41	81	81	81	80	79	82	83	83	84	81	79	84
45	82	80	80	81	83	83	83	81	81	80	79	72
51	83	82	82	83	84	87	85	87	87	88	90	93
55	*	*	*	72	73	76	76	77	75	75	76	74
60	87	89	86	83	87	90	88	85	84	83	86	88
mean	**89**	**89**	**88**	**86**	**86**	**87**	**88**	**89**	**88**	**88**	**87**	**85**
sd	9	11	9	9	9	9	9	9	8	8	8	9

Mean hourly heart rate atenolol group (post-op)

trial no	hour (from midnight) 1	2	3	4	5	6	7	8	9	10	11	12
1	78	74	80	79	72	72	79	83	80	78	77	75
6	78	76	76	77	74	75	74	73	73	73	71	67
7	67	67	66	60	60	60	61	66	73	63	63	60
9	63	64	71	75	74	72	75	75	75	76	77	*
11	64	67	70	71	74	71	84	81	78	75	73	73
13	67	69	68	68	68	68	77	73	73	68	67	64
15	64	65	67	65	63	64	64	68	75	74	67	70
18	83	85	86	89	92	89	102	93	90	91	87	94
26	69	74	78	80	80	78	84	82	73	73	*	79
27	67	66	68	66	70	72	69	67	72	*	77	77
29	78	78	78	77	78	80	85	85	82	79	73	73
34	91	90	90	90	90	95	96	89	92	88	81	85
36	61	62	63	65	72	71	66	75	65	71	69	72
38	62	60	60	67	64	68	71	74	71	65	62	*
52	64	63	66	68	67	70	74	69	70	68	71	68
56	72	71	75	77	78	78	81	76	80	80	*	78
58	71	72	72	73	75	75	77	76	76	73	72	71
59	52	50	50	48	50	53	49	54	54	62	54	50
mean	**69**	**69**	**71**	**72**	**72**	**73**	**76**	**75**	**75**	**74**	**71**	**72**
sd	9	10	9	10	10	10	13	9	9	8	8	10

trial no	hour (from midnight) 13	14	15	16	17	18	19	20	21	22	23	24
1	80	79	84	79	76	83	87	83	79	82	81	80
6	72	82	82	76	74	77	74	77	89	86	84	85
7	60	68	72	71	66	74	71	70	71	72	68	70
9	*	73	78	80	78	83	83	83	80	75	70	64
11	78	74	77	76	81	86	81	76	75	76	73	68
13	68	75	75	74	71	75	77	*	75	72	70	68
15	70	75	77	74	74	74	72	72	74	75	73	64
18	96	101	96	96	*	92	100	92	87	85	86	81
26	80	79	82	83	79	82	81	77	72	72	73	70
27	79	75	77	79	74	83	79	70	69	69	70	67
29	74	79	80	82	82	83	86	87	86	79	78	79
34	87	89	90	89	91	93	95	97	96	95	92	92
36	68	74	77	79	78	69	81	81	79	70	74	65
38	64	64	64	64	66	71	75	73	71	68	65	64
52	*	66	69	70	70	70	71	72	71	67	66	62
56	81	82	81	80	83	83	86	87	87	80	77	74
58	74	82	83	82	81	84	87	85	81	74	75	72
59	52	54	57	56	54	54	54	52	54	60	56	55
mean	**74**	**76**	**77**	**77**	**75**	**78**	**80**	**78**	**77**	**75**	**74**	**71**
sd	11	10	9	9	9	10	10	11	10	8	8	9

Hourly ectopic rate - xamoterol group (pre-op)

	hour (from midnight)											
trial no	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**
4	2	3	8	4	4	3	3	10	9	5	10	6
8	12	8	3	4	3	5	*	*	*	*	*	*
10	189	262	137	249	100	111	220	293	*	*	*	*
12	0	0	0	0	0	0	0	0	4	0	0	0
14	0	0	0	0	0	0	12	5	0	0	0	*
16	1	0	1	1	0	1	1	4	1	2	0	1
17	15	14	8	55	47	32	48	56	57	26	33	38
22	1	0	0	0	1	0	0	0	0	0	0	0
30	1	0	0	1	0	1	0	1	0	0	0	3
33	0	0	0	0	0	0	0	0	0	0	*	*
35	12	13	9	13	9	10	15	19	11	12	15	12
45	1	1	0	0	2	0	0	0	0	0	1	2
51	0	4	10	6	19	54	23	36	21	29	5	22
57	36	86	81	57	68	42	48	41	56	19	9	20
60	2	13	7	10	6	5	16	18	18	13	10	6
median	**1**	**3**	**3**	**4**	**3**	**3**	**7.5**	**7.5**	**4**	**2**	**3**	**6**
Q1	0	0	0	0	0	0	0	0	0	0	0	1
Q3	12	13	9	13	19	32	29.3	37.2	19.5	16	10	20

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
4	10	2	4	4	2	76	2	7	3	11	1	25
8	*	*	*	*	*	16	3	0	2	2	*	5
10	*	380	365	344	365	376	388	249	261	438	296	8
12	0	0	0	0	0	0	0	0	0	0	0	0
14	*	1	0	1	0	3	2	0	0	0	1	0
16	1	1	1	0	1	2	0	0	0	0	0	1
17	79	106	47	46	20	63	86	158	55	63	40	5
22	10	6	0	0	0	0	0	2	0	0	0	0
30	2	0	0	0	0	0	0	0	0	0	1	0
33	0	0	0	0	0	0	0	0	0	0	0	0
35	18	31	20	*	21	23	22	23	14	17	14	5
45	1	0	1	1	0	3	1	0	0	0	0	0
51	7	38	22	45	45	7	59	96	74	64	47	47
57	26	45	10	24	47	50	53	27	13	34	34	30
60	14	20	23	19	21	20	19	17	9	12	13	6
median	**8.5**	**4**	**2.5**	**1**	**1.5**	**7**	**2**	**2**	**2**	**2**	**1**	**5**
Q1	1	0	0	0	0	0	0	0	0	0	0	0
Q3	17	39.8	22.2	34.5	27	50	53	27	14	34	35.5	0

Hourly ectopic rate - atenolol group (pre-op)

	hour (from midnight)											
trial no	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	6	14	7	4	7	9	7	9	72	28	11	34
6	8	4	8	8	1	8	5	6	8	1	2	4
7	0	0	0	0	12	19	*	*	*	*	*	*
9	2	2	2	3	6	2	9	12	30	21	17	7
11	21	6	12	25	17	13	28	38	27	20	23	*
13	0	0	1	0	0	1	0	0	0	2	2	0
15	14	17	11	19	8	6	9	7	9	12	*	*
18	0	0	0	0	0	0	0	5	1	2	4	0
19	0	0	1	0	1	0	2	2	2	0	0	0
23	0	3	3	1	2	3	21	16	4	2	0	1
27	7	8	3	0	0	1	20	1	0	2	*	*
29	0	0	0	0	0	0	3	9	2	1	0	1
34	0	0	0	0	0	0	0	0	0	0	0	0
36	1	0	0	0	0	0	0	0	1	0	0	0
38	0	2	4	0	1	1	0	2	1	1	0	0
42	0	0	0	0	0	1	1	2	1	0	0	2
43	0	0	0	0	0	3	0	1	2	1	2	1
52	1	0	0	0	0	0	0	0	1	1	1	*
58	12	0	0	2	0	1	0	2	10	0	4	1
median	**0**	**0**	**1**	**0**	**0**	**1**	**1.5**	**2**	**2**	**1**	**1.5**	**1**
Q1	0	0	0	0	0	0	0	0.75	1	0	0	0
Q3	7	4	4	3	6	6	9	9	9.25	4.5	4	2.5

Hourly ectopic rate - atenolol group (pre-op) (continued)

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	69	71	21	12	30	154	205	231	101	36	20	9
6	*	8	2	5	2	9	12	8	8	3	5	10
7	13	33	20	49	22	14	49	16	27	14	17	35
9	1	0	5	6	8	6	1	7	7	3	2	6
11	*	10	11	6	7	15	14	13	5	9	12	4
13	0	0	1	1	0	0	0	0	0	0	0	0
15	*	*	*	*	*	*	43	2	49	11	10	8
18	2	2	0	1	0	0	0	0	0	0	2	0
19	0	0	0	0	1	0	1	0	0	0	0	0
23	1	0	0	0	0	0	0	0	2	0	0	4
27	2	0	4	1	2	4	2	7	2	5	3	2
29	0	0	0	0	1	0	0	1	0	0	1	0
34	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0
38	1	0	3	0	1	0	2	1	1	0	0	0
42	1	0	1	0	1	1	0	0	2	1	0	1
43	0	2	0	2	0	2	0	2	0	1	0	0
52	0	0	0	0	1	0	0	0	1	0	0	0
58	2	0	*	8	11	7	42	1	4	6	7	18
median	**1**	**0**	**1**	**1**	**1**	**0.5**	**1**	**1**	**2**	**1**	**1**	**1**
Q1	0	0	0	0	0	0	0	0	0	0	0	0
Q2	2	3.5	4.5	6	7.25	7.5	14	7	7	6	7	8

Hourly ectopic rate - xamoterol group (post-op)

	hour (from midnight)											
trial no	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
4	1	3	2	2	8	5	0	9	5	4	3	1
8	0	0	0	0	0	3	0	2	1	2	2	1
10	34	8	80	21	108	158	216	174	84	75	57	98
12	0	0	0	0	0	0	0	0	0	0	1	0
14	0	0	0	0	0	0	0	0	0	0	0	*
16	103	105	120	92	58	67	53	72	95	91	*	*
17	41	45	3	26	58	27	74	71	35	*	*	*
22	0	2	0	0	0	1	2	0	20	0	0	0
25	15	20	21	13	16	13	23	24	19	22	15	19
30	0	0	0	0	1	1	0	0	6	5	6	8
33	0	0	0	0	0	0	0	0	0	0	0	*
35	1	1	1	7	2	3	6	18	1	4	0	3
40	0	0	0	0	1	0	0	0	0	0	0	*
41	0	0	0	0	0	0	0	0	0	0	*	0
45	0	0	0	0	1	3	9	*	7	12	10	17
51	7	1	16	13	11	20	34	12	6	*	*	*
55	1	1	1	0	2	2	3	1	0	3	4	*
60	17	26	34	31	30	24	22	16	11	9	7	18
median	**0.5**	**1**	**0.5**	**0**	**1.5**	**3**	**2.5**	**2**	**5.5**	**3.5**	**2.5**	**3**
Q1	0	0	0	0	0	0	0	0	0	0	0	0
Q2	15.5	11	17.3	15	19.5	21	25.7	21	19.3	11.3	7.75	18

Hourly ectopic rate - xamoterol group (post op) (continued)

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
4	*	3	1	0	3	0	1	3	0	0	1	1
8	1	0	0	3	0	4	1	10	4	16	1	0
10	283	275	175	136	92	51	33	13	5	11	89	139
12	0	0	1	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	1	0
16	26	7	70	93	50	49	84	50	17	14	26	96
17	*	*	*	6	2	10	5	2	7	1	11	60
22	0	0	2	0	0	4	1	4	2	1	1	0
25	11	12	12	19	17	32	21	20	16	19	23	11
30	6	6	0	2	3	3	6	1	1	7	2	3
33	0	0	0	0	0	0	0	1	0	0	0	0
35	*	0	0	1	1	1	1	0	1	2	3	0
40	0	1	0	0	0	0	0	0	0	0	0	0
41	0	0	0	1	0	0	2	0	0	1	0	0
45	17	13	1	2	0	1	3	2	2	1	0	0
51	2	1	2	10	13	6	17	37	6	63	12	65
55	*	*	*	2	1	0	3	0	0	0	0	0
60	26	18	19	23	21	32	26	27	32	22	33	33
median	**1.5**	**1**	**1**	**2**	**1**	**2**	**2.5**	**2**	**1.5**	**1**	**1**	**0**
Q1	0	0	0	0	0	0	0.75	0	0	0	0	0
Q2	19.3	10.8	9.5	12.3	14	15.5	18	14.8	6.3	14.5	14.8	39.8

Hourly ectopic rate - atenolol group (post-op)

	hour (from midnight)											
trial no	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	41	14	26	44	52	24	55	17	57	37	10	241
6	0	3	1	1	1	0	0	3	2	2	2	1
7	746	706	556	12	61	77	1	6	5	0	0	0
9	0	0	0	0	0	0	0	0	1	0	1	*
11	0	0	1	0	0	1	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0
15	6	7	2	3	3	3	7	7	11	7	2	15
18	0	0	0	0	0	0	0	0	0	0	1	1
26	2	0	1	3	3	0	3	2	4	5	*	3
27	44	24	18	23	16	17	6	1	4	*	0	4
29	0	1	1	0	0	0	0	0	0	0	1	0
34	0	1	0	0	3	3	3	1	0	0	0	0
36	1	0	1	0	0	0	0	0	0	0	0	0
38	3	1	0	1	2	20	2	0	0	1	2	*
52	0	0	3	1	0	0	0	0	0	0	0	0
56	8	11	5	4	0	0	1	2	2	1	*	7
58	0	0	0	1	0	0	0	0	0	2	2	2
59	1	0	0	1	0	1	0	0	0	0	5	0
median	**0.5**	**0.5**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0.5**
Q1	0	0	0	0	0	0	0	0	0	0	0	0
Q2	6.5	8	3.5	3.25	3	6.5	3	2.25	4	2	2	3.8

Hourly ectopic rate - atenolol group (post-op) (continued)

	hour (from midnight)											
trial no	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	120	284	97	166	137	63	67	19	4	12	11	79
6	1	0	0	0	0	0	0	0	0	0	4	2
7	1	1	626	531	275	481	749	659	624	753	814	819
9	*	0	0	0	0	0	0	0	0	0	0	0
11	1	1	1	0	0	0	0	0	0	0	0	1
13	0	0	2	0	2	1	1	*	0	2	0	0
15	8	15	12	5	13	11	7	16	23	8	12	11
18	0	0	0	0	*	0	0	0	0	0	0	0
26	4	1	0	0	1	2	1	0	1	5	1	0
27	14	1	2	1	4	3	0	1	0	0	18	36
29	1	0	0	1	0	1	0	0	1	0	0	2
34	0	0	1	2	2	0	3	1	0	2	1	0
36	0	0	0	0	0	0	0	0	0	0	0	0
38	0	1	1	2	2	0	2	2	3	0	1	0
52	*	0	1	1	1	0	1	0	2	0	0	0
56	0	0	0	0	1	0	1	0	1	0	1	7
58	0	0	1	0	0	1	4	1	2	1	0	0
59	0	1	0	0	1	1	1	0	0	0	0	0
median	**0.5**	**0**	**1**	**0**	**1**	**0.5**	**1**	**0**	**0.5**	**0**	**0.5**	**0**
Q1	0	0	0	0	0	0	0	0	0	0	0	0
Q2	3.25	1	2	2	3	2.2	3.3	1.5	2.2	2.7	5.7	8

Ectopic count per 24 hours - xamoterol group

	pre-op			post-op		
trial no	**ectopics recorded**	**hours recorded**	**ectopics per 24hrs**	**ectopics recorded**	**hours recorded**	**ectopics per 24 hrs**
4	214	24	214	56	23	58
8	63	12	126	51	24	51
10	5031	19	6355	2415	24	2415
12	4	24	4	2	24	2
14	25	22	27	1	23	1
16	20	24	20	1438	22	1569
17	1197	24	1197	484	18	645
22	20	24	20	40	24	40
25	*	*	*	433	24	433
30	10	24	10	67	24	67
33	0	22	0	1	23	1
35	358	23	374	57	23	59
40	*	*	*	2	23	2
41	*	*	*	4	23	4
45	14	24	14	101	23	105
51	780	24	780	354	21	405
55	*	*	*	24	20	29
57	956	24	956	*	*	*
60	317	24	317	557	24	557
		median	**126**		**median**	**59**
		Q1	14		Q1	4
		Q3	780		Q3	464

Ectopic count per 24 hours - atenolol group

	pre-op			post-op		
trial no	**ectopics recorded**	**hours recorded**	**ectopics per 24 hrs**	**ectopics recorded**	**hours recorded**	**ectopics per 24 hrs**
1	1167	24	1167	1677	24	1677
6	135	23	141	23	24	23
7	340	18	453	8503	24	8503
9	165	24	165	2	22	2
11	336	22	367	6	24	6
13	8	24	8	8	23	8
15	235	16	353	214	24	214
18	19	24	19	2	23	2
19	10	24	10	*	*	*
23	63	24	63	*	*	*
26	*	*	*	42	23	44
27	76	22	83	237	23	247
29	19	24	19	9	24	9
34	0	24	0	23	24	23
36	2	24	2	2	24	2
38	21	24	21	46	23	48
42	15	24	15	*	*	*
43	19	24	19	*	*	*
52	6	23	6	10	23	10
56	*	*	*	52	23	54
58	138	23	144	17	24	17
59	*	*	*	12	24	12
		median	**21**		**median**	**20**
		Q1	10		Q1	8
		Q3	165		Q3	94

Post operative diuretic requirement

Data given as mg frusemide. One patient received 1 mg of bumetanide on one occasion, which was assumed to be equivalent to 40 mg frusemide.

Xamoterol group

trial no	day 0	day 1	day 2	day 3	day 4	day 5	total	mean
4	0	40	40	0	0	0	80	13.33
8	10	40	40	40	40	40	210	35.00
10	0	40	40	40	40	0	160	26.67
12	0	40	80	80	80	80	360	60.00
14	0	10	0	0	0	0	10	1.67
16	0	80	40	40	40	0	200	33.33
17	20	80	40	40	40	40	260	43.33
22	20	60	80	80	40	40	320	53.33
25	0	40	40	40	40	40	200	33.33
30	0	60	40	80	80	80	340	56.67
33	50	0	0	0	0	0	50	8.33
35	0	40	40	40	40	0	160	26.67
40	20	60	40	40	40	40	240	40.00
41	20	100	80	80	80	40	400	66.67
45	20	120	80	80	40	40	380	63.33
51	0	40	40	40	40	40	200	33.33
55	70	40	40	40	40	40	270	45.00
57	0	40	40	40	40	40	200	33.33
60	0	20	0	0	0	80	100	16.67
							mean	**36.32**
							sem	4.25

Atenolol group

trial no	day 0	day 1	day 2	day 3	day 4	day 5	total	mean
1	0	40	40	80	80	80	320	53.33
6	0	20	40	40	40	40	180	30.00
7	0	20	40	40	40	40	180	30.00
9	0	40	40	40	40	40	200	33.33
11	0	80	40	40	40	40	240	40.00
13	0	100	80	80	40	40	340	56.67
15	0	30	0	40	40	40	150	25.00
18	0	20	0	0	40	40	100	16.67
19	0	50	40	40	40	0	170	42.50
23	30	100	40	40	40	40	290	48.33
26	0	100	80	80	80	80	420	70.00
27	0	40	40	40	40	40	200	33.33
29	0	60	40	80	240	160	580	96.67
32	10	60	40	40	80	40	270	45.00
34	0	100	80	80	40	40	340	56.67
36	20	70	0	0	0	0	90	15.00
38	0	110	100	80	80	80	450	75.00
42	0	140	80	80	40	40	380	63.33
52	0	100	80	80	80	80	420	70.00
56	0	100	80	80	80	40	380	63.33
58	0	140	100	80	80	40	440	73.33
59	0	100	80	80	80	80	420	70.00
							mean	**50.34**
							sem	4.53

APPENDIX 2

Biochemical results of clinical study

Patient details

Xamoterol group

Trial No	Sex	Age	Ischaemia score	LVEF - angio	LVEF - MUGA	Ca antagonist
4	m	51	12.3	55	60	no
8	m	56	12.2	53	52	yes
10	m	52	13.7	40	52	no
12	m	42	12.4	75	64	no
14	m	54	5.8	62	59	yes
16	f	61	11.1	74	58	yes
17	m	59	10.8	45	56	yes
22	m	54	9.5	65	57	no
25	m	58	12.8	41	51	yes
30	m	56	12.8	65	53	no
33	m	44	10.4	60	68	yes
35	m	66	13.5	50	59	yes
40	m	54	8.4	60	64	yes
41	m	55	11.3	64	67	no
45	m	65	9.5	64	59	yes
51	m	63	11.2	67	61	no
55	m	59	12.5	50	58	yes
57	m	64	12.7	37	36	yes
60	m	62	12.8	58	65	yes

Atenolol group

Trial No	Sex	Age	Ischaemia score	LVEF - angio	LVEF - MUGA	Ca anta-gonists
6	m	47	11.3	46	62	y
7	m	61	8.5	61	66	y
9	m	49	11.7	54	53	y
11	m	58	13.2	53	69	n
13	m	50	10.3	61	56	n
15	m	60	13.4	57	49	y
18	m	52	9.3	67	52	n
19	m	69	11.3	65	64	y
23	m	61	9.8	66	63	y
26	m	51	10.3	70	56	y
27	m	49	10.4	60	66	y
29	f	39	9.3	66	69	y
32	f	54	12.6	58	69	n
36	m	66	10.2	70	62	y
38	m	60	11.2	62	58	n
42	m	51	14.1	48	58	y
43	m	64	10.1	62	58	y
52	m	54	11.3	70	58	y
56	m	59	12.4	56	54	y
58	m	54	13.0	65	48	y
59	m	46	10.6	72	65	n

Receptor densities

Xamoterol group

	Receptor density (fmols/mg protein)			
trial no	**total**	**β_1**	**β_2**	**K_d (pM)**
4	67.05	46.51	20.54	32.58
8	65.33	46.75	18.58	36.62
10	70.54	51.46	19.08	30.40
12	73.81	49.34	24.47	65.00
14	48.26	33.52	14.74	25.76
16	65.81	44.06	21.75	26.53
17	54.44	37.00	17.44	20.42
22	73.75	48.36	25.39	21.05
25	56.39	38.17	18.22	24.95
30	69.09	47.41	21.68	20.79
33	55.70	37.91	17.79	20.43
35	85.82	60.13	25.69	40.15
40	55.19	37.39	17.80	24.32
41	67.07	45.95	21.12	24.37
45	71.72	52.44	19.28	40.35
51	69.80	44.00	25.80	26.37
55	50.79	32.17	18.62	21.67
57	46.26	29.72	16.54	32.26
60	57.27	42.16	15.11	28.49
mean	**63.37**	**43.39**	**19.98**	**29.61**
sem	2.37	1.78	0.79	2.44

Atenolol group

	receptor density (fmols/mg protein)			
trial no	**total**	**β_1**	**β_2**	**K_d (pM)**
6	54.50	34.51	19.99	31.33
7	71.70	50.94	20.76	26.41
9	83.90	53.99	29.91	32.50
11	63.01	43.02	19.99	39.93
13	74.87	49.31	25.56	31.90
15	66.70	44.39	22.31	32.04
18	88.17	60.79	27.38	24.75
19	63.17	43.30	19.87	22.45
23	54.32	37.75	16.57	26.47
26	50.57	34.65	15.92	28.40
27	88.71	59.62	29.09	25.69
29	63.98	43.12	20.86	21.32
32	62.60	38.45	24.15	15.24
36	46.78	32.31	14.47	64.34
38	58.61	36.73	21.88	38.75
42	69.43	47.57	21.86	32.91
43	68.14	51.20	16.94	29.63
52	45.91	30.08	15.83	23.12
56	55.38	34.68	20.70	23.38
58	91.66	59.44	32.33	30.36
59	61.72	42.36	19.36	28.90
mean	**65.90**	**44.20**	**21.70**	**29.99**
sem	2.93	2.03	1.06	2.12

Adenylate cyclase activity

(pmols cAMP/mg/min)

Xamoterol group

trial no	basal	basal (+ GTP)	isoprenaline (10 μM)	procaterol (10 μM)	forskolin (20 μM)
4	2.24	10.06	61.07	76.72	217.96
8	2.39	10.48	46.9	62.83	143.44
10	3.29	14.48	62.31	78.10	353.11
12	2.97	9.96	52.17	60.32	175.24
14	2.89	10.47	72.89	88.03	211.85
16	3.63	14.52	80.39	92.92	298.13
17	2.46	10.46	55.29	65.66	252.94
22	4.11	15.74	78.32	99.11	308.69
25	2.31	7.50	61.11	75.41	264.54
30	2.05	5.51	44.58	55.87	141.89
33	2.15	8.42	43.12	51.95	211.70
35	1.43	3.99	34.71	48.75	129.72
40	1.76	6.10	35.84	46.68	174.69
41	1.98	7.94	43.67	58.91	222.41
45	2.54	12.10	47.88	59.22	231.49
51	1.78	10.02	62.82	72.67	247.19
55	1.24	7.32	39.76	49.89	138.44
57	2.43	9.99	37.30	46.74	163.12
60	1.46	7.11	31.98	41.86	115.20
mean	**2.37**	**9.59**	**64.82**	**52.22**	**210.6**
sem	0.17	0.72	3.82	3.38	15.2

Atenolol group

trial no	basal	basal (+ GTP)	isoprenaline (10 μM)	procaterol (10 μM)	forskolin (20 μM)
6	3.35	12.44	95.64	116.74	304.33
7	3.34	10.74	77.22	85.03	201.34
9	5.69	18.50	92.97	112.29	433.43
11	3.42	15.48	74.83	91.20	314.79
13	4.38	17.69	88.52	109.59	306.83
15	4.03	12.23	76.87	91.13	290.58
18	4.36	14.51	98.08	114.42	445.41
19	3.82	14.31	68.71	84.69	304.36
23	3.63	12.70	69.06	80.55	227.79
26	2.56	9.14	43.80	55.68	214.10
27	4.44	14.85	102.37	115.28	415.92
29	3.52	10.62	74.71	100.53	291.38
32	5.59	17.76	86.75	101.49	423.28
36	2.28	9.90	51.01	69.29	267.86
38	3.10	11.28	90.35	94.23	335.79
42	2.34	12.51	64.33	77.58	327.68
43	2.98	13.65	79.56	91.26	336.23
52	1.86	8.18	44.64	52.61	194.63
56	3.39	12.35	59.33	74.58	261.67
58	3.55	11.92	98.87	111.76	340.83
59	2.26	8.85	52.73	67.91	225.96
mean	**3.52**	**12.84**	**90.37**	**75.73**	**307.8**
sem	0.22	0.64	4.25	3.96	16.4

APPENDIX 3

Animal study

β adrenoceptor subtype density

(fmols/mg/min)

Isoprenaline group

no	Bmax	Kd	% β_1	β_1 Bmax	β_2 Bmax
1	14.69	35.36	84.9	12.47	2.22
2	14.73	41.68	79.5	11.71	3.02
4	16.37	47.64	79.9	13.08	3.29
5	16.38	45.22	83.1	13.61	2.77
10	14.83	44.69	80.3	11.91	2.92
11	12.55	53.26	87.3	10.96	1.59
mean	**14.93**	**44.64**	**82.5**	**12.29**	**2.64**
sd	1.41	5.98	3.2	0.97	0.62

Xamoterol group

no	Bmax	Kd	% β_1	β_1 Bmax	β_2 Bmax
7	26.18	62.08	72.7	19.03	7.15
8	22.42	36.92	71.0	15.92	6.50
13	22.21	42.22	72.8	16.17	6.04
14	19.60	29.61	72.2	14.15	5.45
16	18.33	31.02	73.9	13.55	4.78
17	24.62	39.59	74.6	18.37	6.25
mean	**22.23**	**40.24**	**72.9**	**16.20**	**6.03**
sd	2.95	11.75	1.3	2.19	0.83

β adrenoceptor subtype density (continued)

Sham operated

no	Bmax	Kd	% β_1	β_1 Bmax	β_2 Bmax
3	25.14	41.21	71.2	17.90	7.24
6	23.91	41.05	73.9	17.67	6.24
9	22.30	31.27	66.9	14.92	7.38
12	26.55	58.49	69.9	18.56	7.99
15	25.13	33.73	72.4	18.19	6.94
18	26.31	38.71	71.8	18.89	7.42
mean	**24.89**	**40.74**	**71.0**	**17.69**	**7.20**
sd	1.59	9.57	2.4	1.43	0.58

Adenylate cyclase activities

(pmols cAMP/mg/min)

Isoprenaline group

no	basal	procaterol (10 μM)	isoprenaline (10 μM)	forskolin (20 μM)	% β_1
1	8.18	12.06	26.29	102.55	70.6
2	7.68	10.53	21.12	92.70	67.5
4	7.16	10.86	21.36	80.79	71.4
5	6.88	12.82	23.84	95.09	70.6
10	5.99	9.13	19.13	69.88	72.0
11	5.42	8.85	19.89	69.36	66.0
mean	**6.89**	**10.71**	**21.94**	**85.06**	**69.7**
sd	1.03	1.57	2.67	13.86	2.4

Xamoterol group

no	basal	procaterol (10 μM)	isoprenaline (10 μM)	forskolin (20 μM)	% β_1
7	6.37	16.86	38.20	105.70	59.2
8	6.20	16.65	39.72	102.90	57.7
13	6.97	17.03	43.08	115.76	58.6
14	6.86	16.03	36.86	96.21	61.3
16	4.10	12.34	31.6	78.51	63.7
17	3.90	13.09	35.00	97.14	59.1
mean	**5.73**	**15.33**	**37.41**	**99.37**	**59.9**
sd	1.38	2.07	3.95	12.42	2.2

Adenylate cyclase activity (continued)

Sham operated group

no	basal	procaterol (10 μM)	isoprenaline (10 μM)	forskolin (20 μM)	% β_1
3	5.21	17.12	45.28	95.01	69.3
6	6.64	18.76	45.55	105.65	67.8
9	5.69	18.47	47.61	104.12	70.2
12	4.30	14.76	38.40	77.78	64.1
15	6.45	20.92	48.78	119.43	71.2
18	3.60	14.74	41.79	105.05	60.3
mean	**5.32**	**17.46**	**44.57**	**101.17**	**67.2**
sd	1.20	2.43	3.85	13.87	4.2

APPENDIX 4

Example of Green Lane angiography report

RUN 1 LV ANGIO RAO

There is impairment of contractility antero-laterally but the remainder of LV contracts and there is mild MR with normal mitral inflow. Aortic systolic function appears normal. There is calcification in the proximal LAD.

CORONARY ARTERIOGRAPHY

The left main stem gives off average LAD, good sized intermediate artery and average circumflex arteries. The LAD has a moderate sized diagonal branch proximally with moderate and then severe lesions. This fills slowly ante-gradely and there is then a severe lesion in the LAD before the first large septal branch. Beyond this is a muscle bridge, more distally is a further small diagonal branch. There is an area then of LAD which appears of slightly reduced calibre, but I suspect that this is also intra-myocardial, although this is uncertain. The distal vessel is then of good calibre and quality.

The intermediate artery has a lesion proximally which I think is mild towards moderate and the circumflex artery is irregular with only mild disease affecting the two distal branches, the more proximal of which is the larger.

The right coronary artery has a very early arising posterior descending artery with moderate towards severe lesion at its origin and there is only trivial disease affecting the remainder of the branches distally to the inferior surface of LV. These are only small.

SUMMARY

Moderate coronary artery disease, score 8.7; with overall good LV function impaired antero-laterally. If intervention were considered the proximal LAD lesion may be suitable for angioplasty or alternatively, grafts to the LAD and the posterior descending artery. The proximal diagonal branch is a little small for a separate graft. The remainder of the disease appears relatively mild.

CR/PW
15.4.93

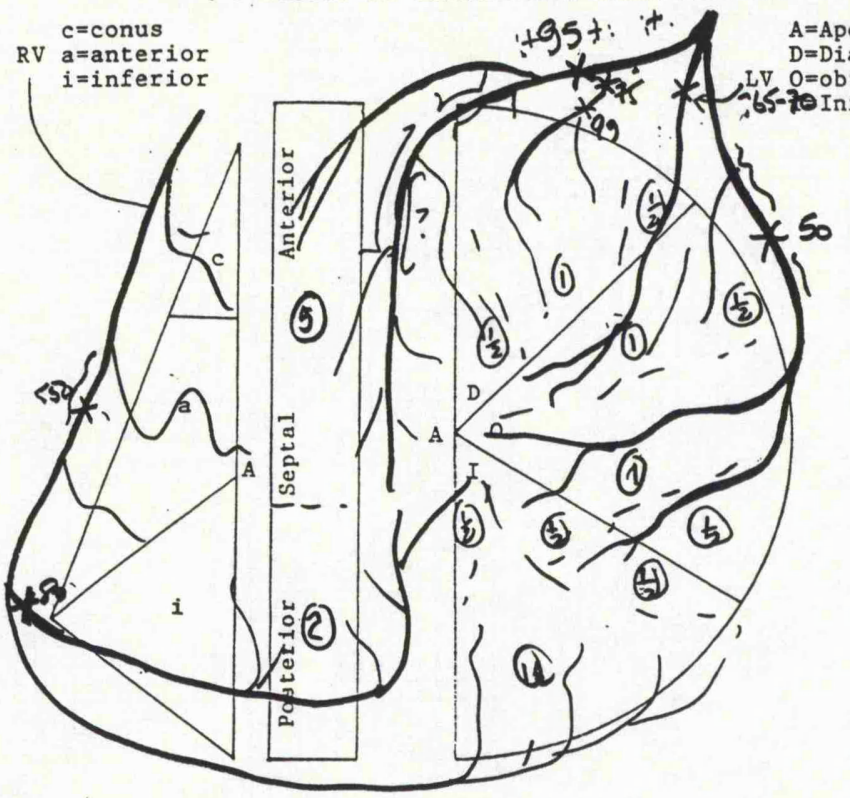
Dr C Reek
Consultant Radiologist

GROBY ROAD HOSPITAL
DEPARTMENT OF CARDIAC RADIOLOGY

Name..... Age.57.years Unit No. .....

Cine No. 564.193. Date..17.3.93.

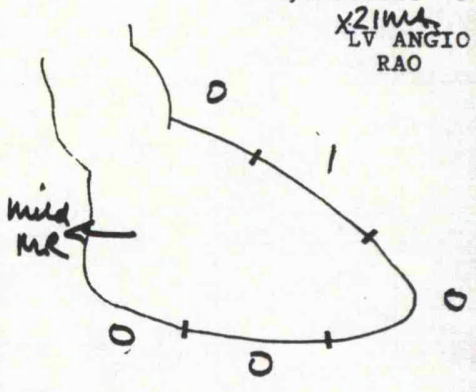
(Stenoses in estimated % cross-section area loss)



Artery	Myoc. Value	Grade	Score	Collat.	Quality Dist.Art.
LAD	5½	b	4.4	/	1
	1	b	0.8		
		Total			
LCA					
CIRC	1½	d	0.6	/	1
	1½ 1	f d d	0.1 1.0		
		Total			
RCA	2½	c	1.5	/	1
	1½	e	0.3		
		Total			
TOTAL	15		8.7		

Normal 0
Hypokinetic 1
Akinetic 2
Dyskinetic 3

x2 IMA.
LV ANGIO
RAO



(After Green Lane Hospital, Auckland, New Zealand

DR. C REEK, DR. N. HUDSON, DR. R KEAL.

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