Br. J. Pharmacol. (1992), **105**, 985–991

Studies on the cardiac actions of flosequinan *in vitro*

[1]R.W. Gristwood, J. Beleta, J. Bou, I. Cardelús, A.G. Fernández, J. Llenas & P. Berga

Division of Biological Sciences, Laboratorios Almirall, Cardener 68-74, Barcelona 08024, Spain

1 We have investigated the *in vitro* cardiac actions of flosequinan and of its major metabolite in man, BTS 53554.

2 Positive inotropic activity was seen with flosequinan in guinea-pig isolated ventricles, the threshold concentration for effect being less than 1×10^{-5} M. BTS 53554 was approximately half as potent as the parent compound.

3 In guinea-pig working whole hearts flosequinan increased left ventricular $dp/dt_{max.}$, indicating a positive inotropic action. This effect was accompanied by increases in heart rate, cardiac output and stroke volume.

4 The virtual complete inhibition of inotropic responses to flosequinan and BTS 53554 by carbachol suggests that these responses are adenosine 3':5'-cyclic monophosphate (cyclic AMP)-mediated.

5 Flosequinan was shown to increase calcium inward current in guinea-pig ventricle, an action consistent with a cyclic AMP involvement in the response.

6 The inotropic activity of flosequinan was not potentiated by the selective phosphodiesterase (PDE) III inhibitor SK&F 94120, a result which indicates that flosequinan does not increase cyclic AMP concentrations via stimulation of adenylate cyclase.

7 Flosequinan inotropic responses were potentiated by rolipram, a selective PDE IV inhibitor, a result consistent with flosequinan being itself a PDE III inhibitor.

8 Biochemical studies with purified enzymes confirmed that flosequinan and BTS 53554 are relatively selective inhibitors of PDE III.

9 A comparison of pharmacological and biochemical data for both flosequinan and BTS 53554 indicates that their PDE III inhibitory potency is sufficient to account for their inotropic activity.

Keywords: Flosequinan; BTS 53554; inotropic activity *in vitro*; phosphodiesterase inhibition

Introduction

Flosequinan (BTS 49465) is an agent currently under development for the treatment of congestive heart failure and hypertension (Cowley *et al.*, 1984; Kessler & Packer, 1987; Sim *et al.*, 1988). Flosequinan was initially described as a vasodilator agent (e.g. Cowley *et al.*, 1984) although recently it has become recognized that the compound has additional positive inotropic properties (e.g. Yates & Hicks, 1988; Falotico *et al.*, 1989; Greenberg & Touhey, 1990) which may have clinical relevance. Clinical studies have shown that flosequinan has an active sulphone metabolite, BTS 53554, which probably contributes to the overall haemodynamic response in man following oral administration (Wynne *et al.*, 1985).

The exact mechanisms of action of flosequinan (or its active metabolite) on the heart and blood vessels are currently unclear. It has been reported that the *in vitro* vasodilator effects of large concentrations of flosequinan are associated with intracellular increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentrations (Allcock *et al.*, 1988) and that it produces weak non selective inhibition of cyclic nucleotide phosphodiesterases (PDEs) in bovine and guinea-pig cardiac tissue, (Frodsham *et al.*, 1989; 1990) and in guinea-pig vascular tissue (Yates, 1991). The positive inotropic responses in dogs *in vivo* persist after β-adrenoceptor blockade (Falotico *et al.*, 1989) implying a mode of action independent of β-receptor stimulation.

The purpose of the present study was to investigate further the effects of flosequinan on cardiac function in guinea-pigs and then to examine effects on PDE isoenzymes. There are currently known to be at least 5 distinct families of PDE isoenzymes: PDEs I to V, these differ in their substrate specificity and affinity as well as in their regulatory properties (see Beavo & Houslay, 1990). Some of the studies were also carried out with the metabolite BTS 53554, two reference PDE III inhibitors, SK&F 94120 and amrinone (Gristwood *et al.*, 1986b) and the non selective PDE inhibitor, 3 isobutyl-1-methylxanthine (IBMX).

Preliminary presentations of some of this work were made to the British Pharmacological Society meetings in London in January and December 1990 (Gristwood *et al.*, 1990; Beleta *et al.*, 1991).

Methods

Inotropic activity in guinea-pig isolated ventricles

Hearts were removed from male guinea-pigs (weight 500–600 g). Strips were dissected from the right ventricles having the dimensions 1 cm long x 1 mm wide (maximum). These were mounted in 30 ml organ baths containing Krebs Henseleit solution gassed with 5% CO_2 in O_2 at 37°C. The preparations were placed under 1 g resting tension whilst electrically stimulated to contract at 1 Hz (threshold voltage +20%). Isometric tension was recorded onto a Letica 4000 polygraph by use of isometric force transducers (Letica TRI 010). Preparations were allowed 30 min to stabilize, prior to drug addition by a cumulative concentration procedure. Drug effects were measured as percentage increases in development of tension (force of contraction) over pre-drug basal values. Inc_{50} values (concentrations causing a 50% increase in force of contraction) were calculated by a least squares regression analysis and s.e.mean values calculated from individual response curves.

[1]Author for correspondence.

Effects in guinea-pig isolated working hearts

Animals were killed 20 min after heparin administration (2000 i.u., i.p.) The heart was quickly excised and placed in a beaker containing Krebs solution at 4°C where any remaining pericardial tissue was removed. The heart was then mounted on the working heart apparatus as described by Flynn *et al.* (1978). When working, Krebs solution (equilibrated with 5% CO_2 in O_2 at 37.0°C) entered the left atrium at a fixed filling pressure of 12.5 cmH_2O and cardiac output was ejected by the left ventricle against a fluid column of height 70 cm. During drug administration by use of a cumulative concentration procedure, the perfusion system was closed, i.e., both coronary and aortic flows were recirculated. Parameters measured were: aortic flow (Skalar 4 mm i.d. electromagnetic flow probe connected to a Skalar MDL 1401 flow meter), coronary flow (Data Logic RC1 drop counter), left intraventricular pressure (LVP) (Druck PDCR 75 pressure transducer), dp/dt_{max}. (Lectromed 5270 differentiator) and heart rate (Lectromed 5250 rate meter triggered by the LVP pressure signal). Responses were recorded directly onto a Lectromed MT8P polygraph and expressed as percentage increases over pre-drug values.

Mechanism of action studies

Effects of carbachol The effects of carbachol were studied on inotropic responses to flosequinan and other drugs in guinea-pig ventricle strips. For this, inotropic responses to the drugs were allowed to stabilize and then carbachol added over the concentration range 1×10^{-8} M to 1×10^{-6} M.

Interaction with isoprenaline The interaction of flosequinan with isoprenaline was studied in guinea-pig ventricle strips. Pairs of strips were obtained from the same ventricle one of which was initially treated with flosequinan 1×10^{-4} M and the other with isoprenaline 3×10^{-9} M. Following stabilization of responses the second drug was then added.

Interaction with other phosphodiesterase isoenzyme selective inhibitors The interaction of flosequinan with a known PDE III inhibitor, SK&F 94120 at 3×10^{-5} M, was studied in guinea-pig ventricle strips. Pairs of strips were obtained from the same ventricle, one of which was treated with SK&F 94120 before addition of flosequinan.

The interaction of drugs with the PDE IV inhibitor, rolipram (Reeves *et al.*, 1987) were also studied. For this, preparations were pretreated with SK&F 94120 3×10^{-5} M, flosequinan 1×10^{-4} M, BTS 53554 or vehicle and allowed 10 min to equilibrate before the addition of rolipram 1×10^{-6} M.

Separation of phosphodiesterase isoenzymes The effects of drugs on purified PDE enzymes were studied. Cyclic nucleotide phosphodiesterases I to IV were obtained from guinea-pig ventricular tissue following the procedure decribed by Reeves *et al.* (1987), except that the chromatographic step was performed with a MONO-Q ion exchange column attached to a Pharmacia FPLC system. A representative elution profile of PDE isoenzyme activities is shown in Figure 1. The isoenzymes were characterized before use in terms of substrate selectivity and affinity and by the effect of calcium ions (10 μM) plus calmodulin (1.2 μM), cyclic GMP and the selective inhibitors rolipram, SK&F 94120 and zaprinast (see Table 1A). Active enzyme fractions were pooled and kept frozen at −20°C in the presence of 1 g l^{-1} bovine serum albumin until used.

Cyclic GMP specific phosphodiesterase (PDE V) was purified from dog platelets. Briefly, blood was freed of red and white cells by differential centrifugation and the platelet rich plasma was washed three times with phosphate buffered saline (10 mM, Na, K phosphate, 140 mM NaCl, EDTA 2 mM, pH 7.4). The pellet was resuspended in 20 mM BisTris pH 6.5 buffer, containing 50 mM sodium acetate, 2 mM benzamidine, 2 mM EDTA, 5 mM β-mercaptoethanol and 50 μM phenylmethylsuphonylfluoride (PMSF), and disrupted by sonication (Branson Sonifier 250, 50% output, 1 min). All procedures were performed at 4°C. The cell lysate was centrifuged at 40000 *g* for 20 min and the supernatant was filtered through a 0.22 μM filter. A volume of 2 ml (corresponding to 10^{10} cells) was chromatographed, characterized and stored following the same procedures described for the guinea-pig enzymes. The preparation of PDE V was known not to be contaminated with PDE I because it was not active when cyclic AMP was used as substrate and was not affected by Ca^{2+} calmodulin.

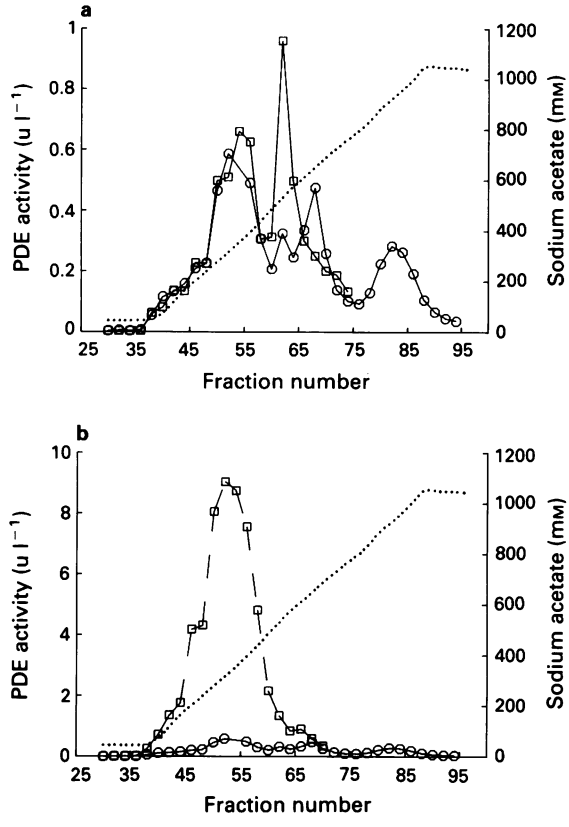

Figure 1 Elution profiles of cyclic nucleotide phosphodiesterase (PDE) activities from guinea-pig ventricular muscle on a MONO Q ion exchange column. The supernatant from the low speed centrifugation was filtered through 0.22 μM filters and 5 to 10 ml were applied at a flow rate of 1 ml min^{-1} to a 1 ml column. This was washed with 15 ml of homogenization buffer and the isoenzymes eluted with a linear gradient of sodium acetate (50–1000 mM) in the same buffer. Fractions (0.5 ml) were collected and assayed for PDE activity. (a) Substrate was 1 μM cyclic AMP in the absence (○) and in the presence of 1 μM cyclic GMP (□). (b) Substrate was 1 μM cyclic AMP in the absence (○) and in the presence of calcium ions and calmodulin (□). The different isoenzymes were identified in the following fractions:: PDE I, 48 to 56; PDE II, 61 to 63; PDE IV, 68 to 70; PDE III, 84 to 87.

Cyclic nucleotide phosphodiesterases were assayed following the procedure of Thompson & Strada (1984). Inhibition assays at 30°C for 20 min were run in duplicate at a substrate concentration of 0.25 μM. The substrate was cyclic AMP for PDE I to IV and cyclic GMP for PDE V. PDE I was assayed in the presence of calcium ions and calmodulin at the above specified concentrations and PDE II was assayed in the presence of 5 μM unlabelled cyclic GMP. For all purified enzymes the slopes of the inhibition curves obtained with drugs were not significantly different from −1.

Table 1 (A) Characterization of the isolated phosphodiesterase (PDE) isoenzymes used

	Phospodiesterase isoenzyme				
	I	II	III	IV	V
K_m cyclic AMP (μM)	1.6	86**	0.3	2.3	>1000
K_m cyclic GMP (μM)	1.8	23***	n.d.	>1000	2.7
Ca^{2+}/calmodulin	15 ± 2.5*	n.e.	n.e.	n.e	n.e.
Cyclic GMP	n.d.	15 ± 3*	7.0 ± 0.17	<3.7 (27)	n.d.
Zaprinast	5.1 ± 0.1	4.2 ± 0.3	<3.7	4.1 ± 0.05	6.8 ± 0.05
SK&F 94120	<3.7 (26)	<3.7 (33)	5.1 ± 0.1	<3.7 (2.5)	<3.7 (30)
Amrinone	<3.7 (13)	<3.7 (43)	4.7 ± 0.2	<3.7 (26)	n.d.
Rolipram	<3.7 (19)	<3.7 (18)	3.9 ± 0.13	6.4 ± 0.06	3.7 ± 0.02
IBMX	5.2 ± 0.04	4.3 ± 0.15	5.3 ± 0.02	5.0 ± 0.03	5.3 ± 0.01
(B) Inhibition of the same isoenzymes by flosequinan and BTS 53554					
Flosequinan	<3.7 (21)	<3.7 (26)	4.2 ± 0.03	<3.7 (16)	<3.7 (38)
BTS 53554	<3.7 (14)	<3.7 (30)	3.9 ± 0.02	<3.7 (16)	<3.7 (33)

For each drug 5–7 concentrations were tested in duplicate for at least 2 different enzyme preparations. Values are $-\log_{10}$ IC_{50} ± s.e.mean except where stated. Numbers in parentheses indicate percentage inhibition at the highest drug concentration tested (200 μM).
n.d. not determined.
n.e. no effect.
* indicates fold activitation.
** indicates $S_{0.5}$ because of non hyperbolic kinetics.
*** $K_m$value for cyclic AMP in the presence of 5 μM cyclic GMP.

Effects on slow inward current Intracellular action potentials along with force of contraction were recorded from guinea-pig papillary muscles by methods previously described (Gristwood *et al.*, 1987).

Preparations were electrically stimulated to contract (0.5 Hz using large supramaximal voltages) in depolarizing Krebs solution at 37°C (see drugs and solutions). Following a 15 min period of equilibration under these conditions flosequinan was administered to the perfusion medium and its effect evaluated at the time of maximal response (10 min later).

Drugs, reagents and solutions

The following drugs were used, SK&F 94120 (5-(4-acetamidophenyl)-pyrazin-2(H1)-one acetamidophenyl; a gift from SK&F Ltd., Welwyn, U.K.), rolipram (a gift from Schering A.G., Germany), zaprinast (a gift from May and Baker, U.K.), amrinone, obtained from Resfar, Italy, isoprenaline sulphate obtained from Boehringer Ingelheim, W. Germany and propranolol hydrochloride obtained from Chemo Iberica, Spain. Flosequinan and the sulphone metabolite BTS 53554 were synthesized in the Department of Chemical Synthesis, Laboratorios Almirall, S.A., Spain.

[8-^{3}H]-adenosine 3':5'-cyclic monophosphate and [8-^{3}H]-guanosine 3':5'-cyclic monophosphate were from Amersham International (Bucks, U.K.). Benzamidine, cyclic AMP, cyclic GMP, calmodulin, IBMX, carbachol and PMSF were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain).

For the pharmacological studies, drugs were prepared as stock solutions of 10^{-2} M. Initial solvents used were: for SK&F 94120 1% NaOH 1 N in water, for flosequinan 10% polyethylene glycol (PEG) 300 in water and for BTS 53554 50% PEG 300 in water. All other drugs were prepared in physiological saline. Dilutions were made with Krebs solution. For the biochemical studies drugs were dissolved in dilute HCl or dimethylsulphoxide (DMSO). Drug vehicles at concentrations employed did not affect enzyme activities.

The composition of the normal Krebs solution used was as follows (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgCl_2$ 1.2, $CaCl_2$ 2.55, NaH_2PO_4 1 and glucose 11.0. For the depolarizing solution used for electrophysiological experiments the potassium ion concentration was elevated from 4.7 to 22 mM with an equimolar reduction in sodium ion concentration. Propranolol 3×10^{-7} M was included in this solution to inhibit effects attributed to endogenous catecholamines that may have been released by the large stimulus intensity.

Statistics

Values are given as mean ± s.e. of mean.

Where relevant, statistical analyses were carried out by Student's *t* test for paired or unpaired data as appropriate.

Results

Inotropic effects on guinea-pig ventricles

Guinea-pig ventricles consistently responded to flosequinan with increases in force of contraction as shown in Figure 2. Responses commenced within 30 s of drug addition and had plateaued by 5 min. Responses were sustained. The threshold concentration for effect was less than 1×10^{-5} M and the Inc_{50} value was 2.9×10^{-5} M ($\pm 1.2 \times 10^{-5}$ M). SK&F 94120 was approximately 7 fold more potent than flosequinan (Inc_{50}, 4×10^{-6} M ± 2.4×10^{-6} M), whilst BTS 53554 was about half as potent as flosequinan (Inc_{50}, 6.0×10^{-5} M ± 0.8×10^{-5} M), as shown in Figure 2. The differences in potencies between all 3 drugs were significant ($P<0.05$).

Propranolol 3×10^{-7} M had no effect on the potency of flosequinan (Inc_{50} value with propranolol 2.1×10^{-5} M ± 0.8×10^{-5} M, $n = 3$).

Effects on guinea-pig isolated working hearts

Immediately prior to administration of flosequinan, values of $dp/dt_{max.}$, heart rate, cardiac output and coronary flow were: 2100 ± 200 mmHg s^{-1}, 226 ± 5 beats min^{-1}, 76 ± 10 ml min^{-1} and 18 ± 2 ml min^{-1} ($n = 4$) respectively. Flosequinan pro-

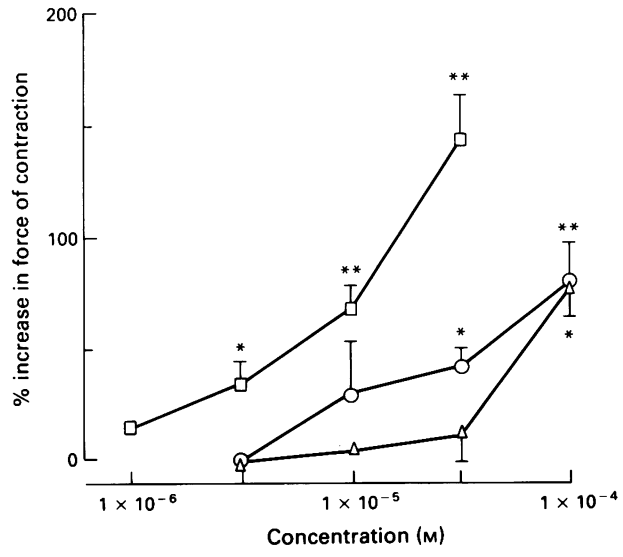

Figure 2 Effects of flosequinan (○), BTS 53554 (△) and SK&F 94120 (□), on the force of contraction of guinea-pig ventricle strips. Basal control values of force of contraction were 82 ± 7 mg, 76 ± 12 mg and 95 ± 8 mg respectively. Points are means of $n = 7$; vertical bars show s.e.mean. Significant responses are indicated $*P<0.05$; $**P<0.001$.

duced significant concentration related increases in $dp/dt_{max.}$, indicating an enhancement of left ventricular contractility (Figure 3). Heart rate and cardiac output were also significantly increased by flosequinan. Coronary flow was little affected at concentrations up to 1×10^{-4} M and a small decrease of this parameter occurred at 1×10^{-3} M.

Although not shown in Figure 3, flosequinan also consistantly caused increases in stroke volume. Thus the mean pre-drug value of stroke volume was 0.34 ± 0.04 ml and after administration of the compound at 1×10^{-4} M was 0.39 ± 0.04 ml (difference significant, $P<0.05$) indicating a mean increase of 15%.

Effects of carbachol on inotropic responses in guinea-pig ventricles

Carbachol caused concentration-dependent inhibition of positive inotropic responses to flosequinan and BTS 53554 (both at 1×10^{-4} M) and SK&F 94120 (3×10^{-5} M), as shown in Figure 4. Carbachol, 1×10^{-6} M, produced a complete reversal of positive inotropic activity to all 3 drugs.

Interaction with SK&F 94120 in guinea-pig ventricles

Flosequinan, 1×10^{-4} M, in untreated preparations caused an 80% ± 15 increase in force of contraction. In the paired preparations SK&F 94120 at 3×10^{-5} M caused a mean 143 ± 21% increase force of contraction and the subsequent addition of flosequinan, 1×10^{-4} M, did not produce a further increase (increase from control remained at 140 ± 18%, $n = 5$). In contrast, responses to isoprenaline, 1×10^{-8} M were markedly larger in the presence of SK&F 94120, increases in the force of the contraction being 82 ± 15% with isoprenaline alone and 220 ± 30% in combination with SK&F 94120, 3×10^{-5} M ($n = 5$).

Interaction with rolipram in guinea-pig ventricles

Rolipram in untreated preparations produced no response, but in preparations treated with flosequinan, BTS 53554 or SK&F 94120 resulted in further inotropic responses as shown in Figure 5, indicating a synergistic interaction with all three drugs. The magnitude of this subsequent rolipram response was greatest in SK&F 94120-treated preparations, intermediate in flosequinan-treated preparations and smallest in the BTS 53554-treated preparations.

Interaction with isoprenaline in guinea-pig ventricles

Flosequinan 1×10^{-4} M or isoprenaline 3×10^{-9} M alone produced increases in force of contraction of 85 ± 29% and 67 ± 17% ($n = 4$) respectively. In combination the 2 drugs caused an increase of 381 ± 58% ($n = 8$) indicating a synergistic interaction.

Studies with isolated cyclic nucleotide phosphodiesterase enzymes

The effects of flosequinan and its metabolite (BTS 53554), on the activity of the five isolated PDE's are shown in Table 1B. It can be seen that both compounds, like SK&F 94120 and amrinone, inhibited PDE type III, although the compounds differed in their potencies against this enzyme with a potency order of SK&F 94120 > amrinone > flosequinan > BTS 53554.

None of the four compounds achieved 50% inhibition of the other PDE isoenzymes at the highest concentration tested. In contrast the non-selective PDE inhibitor IBMX inhibited all isoenzyme types (Table 1A).

Effects of flosequinan on slow response action potentials in guinea-pig ventricles

Flosequinan (1×10^{-4} M), 10 min after administration, produced a stable increase in force of contraction that was accompanied by increases in $dV/dt_{max.}$ and the amplitude and duration of the slow response action potential. These electrophysiological effects of flosequinan were consistently observed in 5 preparations, as shown in Table 2.

Discussion

The results from the present study have confirmed previous findings that flosequinan has positive inotropic activity *in vitro* (Falotico *et al.*, 1989; Greenberg & Touhey, 1990). Thus, increases in ventricular contractility were observed in

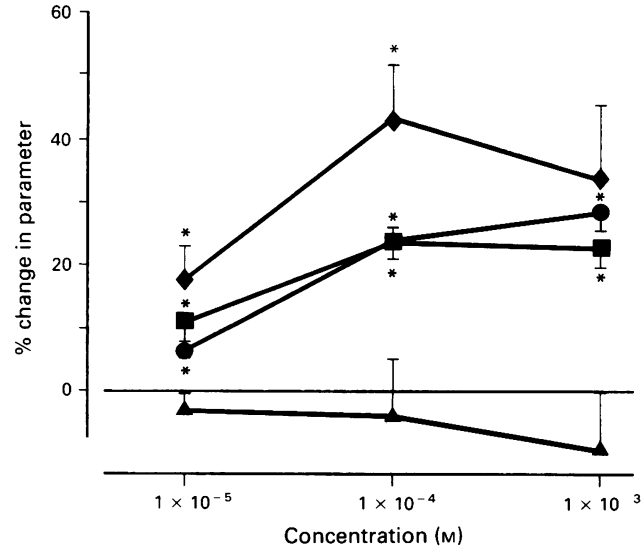

Figure 3 Effects of flosequinan on guinea-pig isolated working hearts. Parameters shown are left ventricular dp/dt_{max} (●), heart rate (■), cardiac output (◆) and coronary flow (▲). Points show mean values with s.e.mean indicated by vertical bars. $*P<0.05$; $**P<0.005$.

Table 2 Effects of flosequinan on slow response action potentials in guinea-pig ventricle

Condition	$dV/dt_{max.}$ ($V\ s^{-1}$)	*Action potential amplitude* (mV)	*Action potential duration* (ms)
Control	7.3 ± 0.8	81.9 ± 3.7	67.2 ± 8.4
Flosequinan 1×10^{-4} M	11.5 ± 1.1*	88.6 ± 3.7*	101.8 ± 9.0*

*$P<0.05$: significantly different from control values; ($n = 5$).

guinea-pig ventricles and in guinea-pig working whole hearts. The increased contractility of the working hearts was associated with a tachycardia and an increase in cardiac output. In addition the results showed that flosequinan can cause an increase in cardiac output via a direct action on the heart. The finding that stroke volume was increased indicates that the enhanced contractility played a part in the output increase.

Concerning mechanism of action: reversal of positive inotropic responses by carbachol has been shown to be selective for cyclic AMP-dependent inotropic responses (Endoh, 1980; Gristwood *et al.*, 1987) and, therefore, represents a relatively simple procedure to test for this. That carbachol at

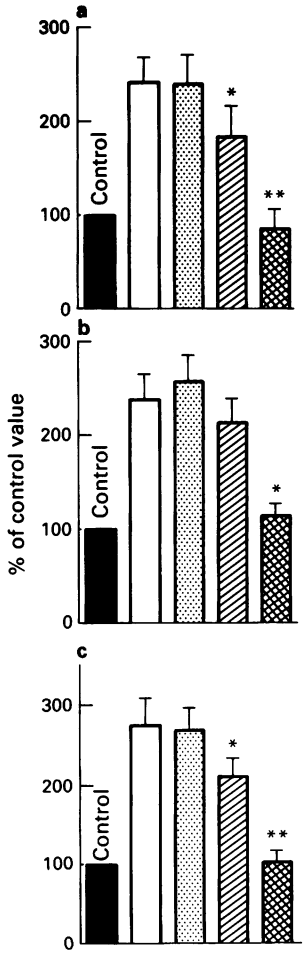

Figure 4 Effects of carbachol at 1×10^{-8} M (▒), 1×10^{-7} M (▨) and 1×10^{-6} M (▩) on inotropic responses to (a) flosequinan 1×10^{-4} M, (b) BTS 53554 1×10^{-4} M and (c) SK&F 94120 3×10^{-5} M. Basal control values of force of contraction prior to drug addition were (a) 83 ± 8 mg, (b) 50 ± 14 mg and (c) 49 ± 10 mg. Values shown are means with s.e.mean shown by vertical bars, $n = 6-7$. *$P<0.05$; **$P<0.005$.

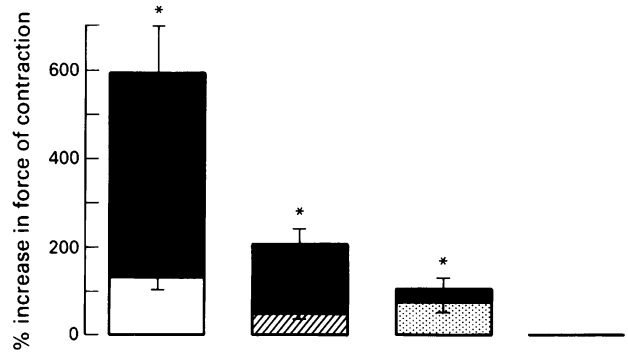

Figure 5 Responses to rolipram 1×10^{-6} M (■) in guinea-pig ventricles pretreated with SK&F 94120 3×10^{-5} M (□), flosequinan 1×10^{-4} M (▨) and BTS 53554 1×10^{-4} M (▒), and also in non pretreated preparations (no response). Basal control values of force of contraction were 72 ± 28 mg, 86 ± 14 mg, 110 ± 5 mg and 70 ± 10 mg respectively. Values are means with s.e.mean shown by vertical bars, $n = 5-8$. *Response to rolipram significant: $P<0.05$.

1×10^{-6} M virtually abolished the inotropic activity of effective concentrations of flosequinan, BTS 53554 and SK&F 94120 is consistent with a major cyclic AMP involvement in their responses. The lack of effect of propranolol on the flosequinan Inc_{50} indicated that these responses were not mediated via β-adrenoceptors.

Selective PDE III inhibitors like SK&F 94120 or amrinone have been previously shown to potentiate the positive inotropic effects of adenylate cyclase stimulants, such as isoprenaline (Gristwood *et al.*, 1987) or histamine (Honerjager *et al.*, 1981) in guinea-pig ventricle. Thus, the lack of synergy between effective inotropic concentrations of flosequinan and SK&F 94120 provided further evidence that flosequinan is not stimulating adenylate cyclase activity via β-adrenoceptors or other cyclase coupled receptors, at these concentrations.

In contrast to the lack of potentiation of flosequinan response by the PDE III inhibitor SK&F 94120, our studies have clearly shown that the inotropic effects of flosequinan are potentiated by the PDE IV inhibitor, rolipram. It was previously reported (Gristwood & Owen, 1986; Gristwood *et al.*, 1986a) that in guinea-pig and cat ventricles there is a specific synergistic interaction on force of contraction between the selective PDE III inhibitor SK&F 94120 and rolipram. Subsequently it was reported in rabbit heart muscle that the simultaneous inhibition of PDE IV potentiated the inotropic activity of PDE III inhibitors, whereas in rat heart neither selective inhibitors of PDE III or IV alone produced an inotropic response but a response was observed when these were administered in combination (see Nicholson *et al.*, 1991). Thus, one explanation for the present results with flosequinan, as well as BTS 53554, is that they are, like SK&F 94120, functional inhibitors of PDE III. Further indirect evidence for PDE inhibitory activity was provided by the finding that flosequinan potentiated inotropic responses to isoprenaline.

The studies with isolated PDE isoenzymes confirmed that flosequinan and BTS 53554, like SK&F 94120, can inhibit cardiac PDE III activity. Furthermore, our data indicate that flosequinan, and to a lesser extent BTS 53554, are relatively selective inhibitors of PDE III. The selectivity profile for flosequinan was similar to that of amrinone, a well known selective PDE III inhibitor currently used for the therapy of congestive heart failure. The oral dose range of amrinone tested clinically (100–400 mg) is similar to that currently used for flosequinan (100–150 mg) and it is interesting that these 2 compounds have very similar PDE III inhibitor potencies as well as oral potencies in man. The absolute selectivity for flosequinan and BTS 53554 cannot be calculated accurately from our data. However, extrapolation of inhibitory data observed (to yield theoretical IC_{50} values) with the other cardiac enzymes studied suggest that flose-

quinan has a selectivity of about 20 fold for PDE III whilst BTS 53554 has a selectivity of about 4 fold.

The order of inotropic potency and PDE III inhibitory potency of SK&F 94120 > flosequinan > BTS 53554, was the same and if one considers the ratio of Inc_{50} to IC_{50} values it is found that the values obtained for flosequinan (1.8) and BTS 53554 (2.0) are very similar to that for SK&F 94120 (1.9). It is this similarity that leads us to conclude that the PDE III inhibitory potencies of flosequinan and BTS 53554 are sufficient to account for their inotropic activity in guinea-pig ventricle.

Our electrophysiological studies in guinea-pig heart provided further evidence for this. Thus, in partially depolarized preparations in which the action potential configuration is due predominantly to I_{si}, flosequinan at 1×10^{-4} M caused large increases in all measured parameters, consistent with an enhancement of I_{si}. This activity which occurred in parallel with the positive inotropic activity is consistent with a cyclic AMP mediated increase in I_{si} (see Honerjager *et al.*, 1981).

An important question is whether, at plasma concentrations achieved clinically, flosequinan acts as a positive inotropic agent. Based on our results, we would argue that the inotropic activity of flosequinan is clinically relevant. Thus, peak blood levels of both flosequinan and subsequently its metabolite at hypotensive doses in man are around 1×10^{-5} M (Yates & Hicks, 1988). At this concentration we saw clear inotropic activity with flosequinan both in guinea-pig ventricles and whole hearts as well as significant inhibition of guinea-pig PDE III isoenzyme (20% inhibition). Our argument is strengthened by the finding that an important feature of PDE III inhibitors is their ability to interact synergistically with β-adrenoceptor stimulation in human isolated heart (see Gristwood *et al.*, 1987). Thus, it is probable that *in vivo*, an interaction of flosequinan and/or BTS 53554 with the sympathetic drive to the myocardium would be important. Other workers have also argued, based on studies in dogs, that the positive inotropic effect significantly contributes to the overall haemodynamic response of flosequinan (Falotico *et al.*, 1989; Greenberg & Touhey, 1990).

In conclusion our studies have confirmed that flosequinan has positive inotropic activity in guinea-pig ventricle. The mechanism of inotropic action appears to be related to PDE III inhibition resulting in increased intracellular concentrations of cyclic AMP and an enhancement of calcium influx into the ventricle. The inotropic potency of flosequinan suggests that an inotropic effect should be expected in man at doses used clinically. The positive inotropic activity was shown to contribute to the increase in cardiac output seen in the guinea-pig whole heart *in vitro*. Such an effect in man would probably play an important role in the haemodynamic improvements seen with flosequinan in CHF patients (Cowley, 1991).

After submission of this paper we became aware of an abstract by Frodsham *et al.* (1991) describing weak and non-selective inhibition by flosequinan and BTS 53554 of guinea-pig ventricular PDE isoenzymes separated by Mono Q FPLC. Although we cannot explain the differences between our study and that of Frodsham *et al.*, it is obvious that a major difference is that of the substrate concentrations used (1 μM, for Frodsham *et al.*, 1991). A concentration of 0.25 μM cyclic AMP, used in the present study, is believed to reflect the basal concentration of substrate within the cell (Reeves *et al.*, 1987) and allows a more precise measurement of IC_{50} values for drugs with relatively low potency and limited solubility.

The authors wish to thank A. Sauret, J. Mañé and J. Diaz-Young for technical assistance, and M.D. Moliné for typing the manuscript.

References

ALLCOCK, A.A., FRODSHAM, G.F. & SIM, M.F. (1988). Effects of flosequinan, a novel arteriovenous dilating agent on cGMP levels in rat isolated aortic strips. *Br. J. Pharmacol.*, **94**, 430P.

BEAVO, J.A. & HOUSLAY, M.D. (1990). *Isoenzymes of Cyclic Nucleotide Phosphodiesterases*. Chichester: John Wiley & Sons.

BELETA, J., BERGA, P., BOU, J., FERNANDEZ, A.G., LLENAS, J. & GRISTWOOD, R.W. (1991). The inotropic potency of flosequinan is consistent with its PDE III inhibition activity. *Br. J. Pharmacol.*, **102**, 209P.

COWLEY, A.J. (1991). Clinical efficacy of flosequinan in heart failure. *Am. Heart J.*, **121**, 983–988.

COWLEY, A.J., WYNNE, R.D. & HAMPTON, J.R. (1984). The effects of BTS 49465 on blood pressure and peripheral arteriolar and venous tone in normal volunteers. *J. Hypertension*, **2**, 547–549.

ENDOH, M. (1980). The time course of changes in cyclic nucleotide levels during cholinergic inhibition of positive inotropic actions of isoprenaline and theophylline in the isolated canine ventricular myocardium. *Arch. Pharmacol.*, **318**, 112–120.

FALOTICO, R., HAERTLEIN, B.J., LAKAS-WEISS, C.S., SALATA, J.L. & TOBIA, A.J. (1989). Positive inotropic and haemodynamic properties of flosequinan, a new vasodilator, and a sulphone metabolite. *J. Cardiovasc. Pharmacol.*, **14**, 412–418.

FLYNN, S.B., GRISTWOOD, R.W. & OWEN, D.A.A. (1978). Characterisation of an isolated working guinea-pig heart including effects of histamine and noradrenaline. *J. Pharmacol. Methods*, **1**, 183–195.

FRODSHAM, G., JONES, R.B. & SIM, M.F. (1989). Effects of flosequinan, a novel arteriovenous dilating agent, and its metabolite on bovine heart phosphodiesterase activity. *Br. J. Pharmacol.*, **98**, 733P.

FRODSHAM, G., JONES, R.B. & SIM, M.F. (1990). Effect of flosequinan and its major metabolite, BTS 53554, on phosphodiesterase (PDE) isoenzymes types I, II and III from guinea-pig ventricle. *Br. J. Pharmacol.*, **100**, 441P.

FRODSHAM, G., DICKINSON, K., HAYES, S.L., JONES, R.B. & YATES, D.B. (1991). Effects of flosequinan and BTS 53554 upon isoenzymes of phosphodiesterase (PDE) isolated from guinea-pig ventricle using Mono Q FPLC. *Br. J. Pharmacol.*, **104**, 172P.

GREENBERG, S. & TOUHEY, G. (1990). Positive inotropy contributes to the haemodynamic mechanism of action of flosequinan (BTS 49465) in the intact dog. *J. Cardiovasc. Pharmacol.*, **15**, 900–910.

GRISTWOOD, R.W. & OWEN, D.A.A. (1986). Effects of rolipram on guinea-pig ventricles in vitro: evidence of an unexpected synergism with SK&F 94120. *Br. J. Pharmacol.*, **87**, 91P.

GRISTWOOD, R.W., SAMPFORD, K.A. & WILLIAMS, T.J. (1986a). The effect of SK&F 94120, rolipram and M&B 22948 on force of contraction and cyclic nucleotide content of cat myocardium. *Br. J. Pharmacol.*, **89**, 573P.

GRISTWOOD, R.W., EDEN, R.J., OWEN, D.A.A. & TAYLOR, E.M. (1986b). Pharmacological studies with SK&F 94120, a novel positive inotropic agent with vasodilator activity. *J. Pharm. Pharmacol.*, **38**, 452–459.

GRISTWOOD, R.W., ENGLISH, T.A.H., WALLWORK, J., SAMPFORD, K.A. & OWEN, D.A.A. (1987). Analysis of responses to a selective phosphodiesterase III inhibitor, SK&F 94120, on isolated myocardium, including human ventricular myocardium from 'end stage' failure patients. *J. Cardiovasc. Pharmacol.*, **9**, 719–727.

GRISTWOOD, R.W., LLENAS, J., BOU, J., PUIG, C. & BERGA, P. (1990). Flosequinan (BTS 49465) behaves as an inhibitor of phosphodiesterase type III. *Br. J. Pharmacol.*, **99**, 18P.

HONERJAGER, P., SCHAFER-KORTING, M. & REITER, M. (1981). Involvement of cyclic AMP in the direct inotropic action of amrinone. Biochemical and functional evidence. *Arch. Pharmacol.*, **318**, 112–120.

KESSLER, P.D. & PACKER, M.D. (1987). Haemodynamic effects of BTS 49465, a new long-acting systemic vasodilator drug, in patients with severe congestive heart failure. *Am. Heart. J.*, **113**, 137–143.

NICHOLSON, C.D., CHALLISS, R.A.J. & SHAHID, M. (1991). Differential modulation of tissue function and therapeutic potential of selective inhibitors of cyclic nucleotide phosphodiesterase isoenzymes. *Trends Pharmacol. Sci.*, **12**, 19–27.

REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535-541.

REUTER, H. (1967). The dependence of slow inward current in Purkinje fibres on the external calcium concentration. *J. Physiol.*, **192,** 479–492.

SIM, M.F., YATES, D.B., PARKINSON, R. & COOLING, M.J. (1988). Cardiovascular effects of the novel arteriovenous dilator agent flosequinan in conscious dogs and cats. *Br. J. Pharmacol.*, **94,** 371–380.

THOMPSON, W.J. & STRADA, S.J. (1984). Cyclic nucleotide phosphodiesterase (PDE) In *Methods of Enzymatic Analysis* ed. Bergmayer H.U. 3rd ed., Vol. IV, pp. 127–134. Weinheim: Verlag Chemie.

WYNNE, R.D., CRAMPTON, E.L. & HIND, I.D. (1985). The pharmacokinetics and haemodynamics of BTS 49465 and its major metabolite in healthy volunteers. *Eur. J. Clin. Pharmacol.*, **28,** 659–664.

YATES, D.B. (1991). Pharmacology of flosequinan. *Am. Heart J.*, **121,** 974–983.

YATES, D.B. & HICKS, G.R. (1988). Effects of flosequinan on guinea-pig atria and on cardiac variables in the anaesthetised dog. *Br. J. Pharmacol.*, **95,** 711P.

(Received July 4, 1991
Revised December 17, 1991
Accepted December 23, 1991)