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Is Ro 03-7894 an irreversible antagonist at β-adrenoceptor sites?

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1 Ro 03-7894 (0.6 mM) produced a non-parallel shift to the right of dose-response curves to (−)-isoprenaline in K^+ depolarized uterine preparations from the guinea-pig. The displacement of the curves was readily reversed by washing. A rightward shift of similar magnitude was also produced by Ro 03-7894 in transmurally stimulated ileal preparations. The relaxant effects of fenoterol in carbachol-contracted guinea-pig tracheal preparations (in the presence of 2 μM atenolol) were not altered by 0.6 mM Ro 03-7894. In the three tissues there was no evidence of a reduction in the maximal inhibitory response to the agonists.

2 In uterine and tracheal preparations, Ro 03-7894 (0.6 mM) depressed contractile responses to exogenous calcium. The depression of responses was enhanced after washout of Ro 03-7894 for 80 min. Contractile responses of ileal preparations to transmural stimulation were also depressed by Ro 03-7894.

3 Concentration-effect curves for the positive inotropic effects of (−)-isoprenaline in guinea-pig left atrial preparations were markedly shifted to the right and the maximum response depressed by 0.6 mM Ro 03-7894. Although the rightward shift of the curves was fully reversed during the 120 min washout period, the maximal responses remained depressed. In similar experiments, Ro 03-7894 produced a washout-resistant depression of inotropic responses to histamine and calcium.

4 The results of radioligand binding studies in left atria using (−)-[^{125}I]-iodocyanopindolol indicated that, when compared to the untreated atria, there was no reduction in the maximal density of binding sites 120 min after washout of 0.6 mM Ro 03-7894.

5 On the basis of the present results it is concluded that Ro 03-7894 induces a non-specific depressant effect on smooth and cardiac muscle preparations during exposure to the drug. This depressant effect persists following washout of the drug. There is no evidence for an irreversible effect of Ro 03-7894 at β-adrenoceptor sites.

Introduction

In previous studies it has been suggested that the compound Ro 03-7894 (1-(5-chloroacetylamino-benzfuran-2-yl)-2-isopropylaminoethanol) behaves as an irreversible, non-competitive antagonist at β-adrenoceptor sites in guinea-pig isolated cardiac tissue (Nicholson & Broadley, 1978; Broadley & Nicholson, 1981; Rankin & Broadley, 1982). The evidence for this classification of the antagonist was based on the observations that it reduced the slope and maxima of positive inotropic and chronotropic concentration-response curves to isoprenaline and orciprenaline without affecting curves established to histamine or calcium (Nicholson & Broadley, 1978). In addition, repeated washing of these isolated cardiac tissue preparations did not restore maximal responses to the β-adrenoceptor agonists. Furthermore, this agent reduced the maximal number of specific [^{3}H]-dihydroalprenolol binding sites in membranes prepared from guinea-pig ventricular tissue and remained bound to the tissue during washing procedures which removed bound propranolol from the membranes (Rankin & Broadley, 1982).

The aim of the present study was to examine the effects of Ro 03-7894 as an irreversible β-adrenoceptor antagonist in tracheal, ileal and uterine preparations from the guinea-pig. However, the complex pharmacological actions of Ro 03-7894 in the smooth muscle preparations led us to re-evaluate its actions in atria and question its 'irreversible' antagonistic actions at β-adrenoceptor sites.

The concentration of Ro 03-7894 used in the present study (0.6 mM) was similar to that used in the initial *in vitro* studies with this agent (Nicholson & Broadley, 1978; Broadley & Nicholson, 1981).

Methods

Guinea-pigs (pretreated with reserpine 1 mg kg^{-1} i.p. 18 h) were killed by a blow to the head. The isolated tissues were suspended under a tension of 0.5 g in an organ bath containing Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25, $MgSO_4$ 1.2, glucose 11.7, NaH_2PO_4 1.2, EDTA 0.1, ascorbic acid 0.1) maintained at 37°C and gassed with 5% CO_2 in O_2. Isometric changes in tension were displayed on a Grass model 7C polygraph using a Grass FTO3c transducer coupled to a Grass 7PI preamplifier. Ileal, carbachol-contracted tracheal preparations and left atria in which histamine was used as an agonist, were bathed in solutions containing cocaine (10 μM), corticosterone or hydrocortisone (100 μM) and phentolamine (10 μM); the other tissues were pretreated with phenoxybenzamine (50 μM, 30 min incubation followed by 6 washes in 30 min), to preclude interference from neuronal and extraneuronal uptake, and α-adrenoceptor stimulation.

In some experiments the tissues were bathed in a K^+-depolarizing solution which was identical in composition to the Krebs solution except that all Na^+ salts were replaced by K^+ salts. EDTA was omitted when Ca^{2+}-free, K^+-depolarizing solutions were used.

Organ bath studies

Tone in tracheal preparations (Mylecharane & Raper, 1973) was induced with carbachol (0.5 μM). To preclude interference from possible β_1-adrenoceptor stimulation, atenolol (2 μM) was present in the bathing solution at all times, and fenoterol was used as the agonist.

In studies where the intramural nerves of isolated ileal preparations were electrically stimulated (0.1 Hz, 2.5 ms, maximal voltage 35–45 V), the agonist used was (−)-isoprenaline. Previously published studies (Williams & Broadley, 1982) as well as those in this laboratory (unpublished observations), indicate the presence of a homogeneous population of β_1-adrenoceptors effecting the response in this preparation.

A K^+-depolarizing solution (with Ca^{2+}) was used to induce tone in uterine preparations taken from stilboestrol-pretreated (0.1 mg kg^{-1} i.p. 24 h) animals. In these studies (−)-isoprenaline was used as the agonist since in this tissue a homogeneous population of β_2-adrenoceptors is involved in the response (Krstew *et al.*, 1982).

Positive inotropic responses to (−)-isoprenaline, calcium or histamine were recorded from electrically stimulated left atrial preparations (2.5 Hz, 1 ms, twice threshold voltage). In other experiments, the effective refractory period of left atria (driven as above) was monitored using a paired pulse technique (Mylecharane & Raper, 1971) in which the interval between the pulses of identical voltage and duration (delivered from a Grass S88 stimulator), was steadily increased until a potentiated contraction was observed.

Experimental protocol

After an initial equilibration period, three control cumulative concentration-effect curves to the agonists were established at 30 min (atrium, ileum) or 45 min (trachea, uterus) intervals. The tissues were then incubated with 0.6 mM Ro 03-7894 for 30 min. In some preparations a curve to the agonist was re-established before removal of Ro 03-7894, whilst in others the tissues were washed twice at the end of the incubation period, and then once every 20 min. Curves to the agonists were re-established at 40, 60, 80 or 120 min after washout.

The time-dependent changes in the reactivity of the tissues were assessed in other experiments by omitting the Ro 03-7894 but otherwise following the same protocol.

Concentration-effect curves were plotted as changes in tension (from resting value) against log concentrations of the agonist. Ro 03-7894-induced alterations in resting tension were taken into account when plotting the concentration-effect curves to the agonists.

Radioligand binding studies

Left atria taken from reserpinized guinea-pigs (1 mg kg^{-1} i.p. 18 h) were cut in two, mounted in organ baths containing Krebs-bicarbonate solution and continuously stimulated as outlined previously (2.5 Hz, 1 ms, twice threshold voltage). The protocol followed with regard to time, phenoxybenzamine treatment and washout procedure was identical to that described in the organ bath studies, except that dose-response curves to (−)-isoprenaline were not established. From each atria, one half was treated with 0.6 mM Ro 03-7894, whilst the other served as a control (i.e. no Ro 03-7894). Following a 30 min incubation period, the tissues were washed once every 20 min for 120 min and then removed from the bath and prepared for the binding assay.

Each half of the left atrium was homogenized in 100 vol. ice cold Krebs-phosphate buffer (composition mM: NaCl 119, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 1.9, glucose 11.7, NaH_2PO_4 1.3, Na_2HPO_4 8.7, pH 7.4)

for 15 s using a Polytron homogenizer (setting 10). The homogenate was centrifuged at 39,000 *g* for 10 min and the pellet resuspended (setting 10, 5 s) in 100 vol. of buffer and centrifuged again. The resulting pellet was resuspended in 300 vol. buffer to obtain the final stock of membrane suspension.

The radioligand used was (−)-[^{125}I]-iodocyanopindolol ([^{125}I]-CYP, approximately 2,000 Ci mmol^{-1}, Amersham). Binding assays were performed in disposable polystyrene tubes in which 150 μl of homogenate was combined with 50 μl of radioligand in Krebs-phosphate buffer containing 0.1 mM GTP, 1 mM ascorbic acid and 0.1 mM EDTA, in a total volume of 250 μl. The tubes were incubated for 70 min at 37°C and the assay terminated by the addition of 8 ml ice-cold buffer followed by rapid filtration through Whatman GF/B filters. Each filter was washed with an additional 8 ml ice-cold buffer. Radioactivity retained on the filters was measured using a Packard γ-Counter (Model 5320) at an efficiency of approximately 46%. Specific binding of the radioligand was defined as the difference in the amount of [^{125}I]-CYP bound in the absence and in the presence of propranolol (1 μM). Specific binding ranged from approximately 70% (at 10 pM) to approximately 40% (at 200 pM) of the total binding observed. The concentrations of radioligand used were 10, 20, 50, 80, 100 and 200 pM and all assays were performed in triplicate.

The binding data were analysed using two computer programmes, EBDA (McPherson, 1983a;b) which performed preliminary Scatchard and Hill analyses and created a file for the second programme LIGAND (Munson & Rodbard, 1980). The latter was used to obtain final parameter estimates.

Drugs used

The drugs used were: (−)-isoprenaline bitartrate (Wyeth); fenoterol hydrobromide (Boehringer-Ingelheim); atenolol and propranolol hydrochloride (Imperial Chemical Industries); Ro 03-7894 [1-(5-chloroacetylaminobenzfuran - 2 - yl) - 2 - isospropylaminoethanol] (Roche); phentolamine hydrochloride and reserpine (Ciba-Geigy); hydrocortisone sodium succinate (Glaxo); phenoxybenzamine hydrochloride (Smith, Kline & French); disodium stilboestrol diphosphate (Bristol); cocaine hydrochloride (Macfarlan Smith); carbachol chloride, corticosterone, atropine sulphate, histamine dihydrochloride and guanosine triphosphate (GTP) (Sigma). Stock solutions of (−)-isprenaline, fenoterol and histamine (all 10 mM) were prepared in 0.01 M HCl; corticosterone (0.1 M) in 95% ethanol containing 1 μl 10 M HCl ml^{-1}, and the remaining drugs in distilled water. Dilutions were made using Krebs-bicarbonate solution (Na^+ or K^+ salts as appropriate) containing 1 mM ascorbic acid. Ro 03-7894 was prepared as a solution 2 mg ml^{-1} in Krebs-bicarbonate solution using ascorbic acid to enhance solubility.

Results

Tracheal smooth muscle

Fenoterol elicited a 90 ± 5% (mean ± s.e.mean $n = 8$) reduction of the carbachol (0.5 μM)-induced increase in tone (1.8 ± 0.3g, mean ± s.e.mean, $n = 8$) in this tissue. The pD_2 value for fenoterol was 6.99 ± 0.15 (mean ± s.e.mean, $n = 8$). At the conclusion of a 30 min incubation with 0.6 mM Ro 03-7894, the carbachol-induced increase in tone was reduced to 0.45 ± 0.02 (mean ± s.e.mean, $n = 4$) of that observed under control conditions. In the presence of Ro 03-7894, fenoterol completely abolished carbachol-induced tone. Figure 1 shows cumulative concentration-effect curves to fenoterol in the absence and presence of Ro 03-7894 and indicates the lack of a rightward displacement of the curve.

Surprisingly, contractions to carbachol were reduced after further washing the preparations for 40 and 80 min. Due to the marked depression observed after washout of the Ro 03-7894, it was not possible to re-establish concentration-effect curves to fenoterol. Figure 2 shows the changes in the height of the carbachol-induced contractions with time. The reduction in the responses to carbachol was not surmountable since increasing the carbachol concentration to 5 μM produced only a small increase in the size of the contraction (Figure 2). Addition of calcium chloride to a Ca^{2+}-free, K^+-depolarizing bathing solution elicited concentration-dependent contractions of the trachea, which were not affected by the presence of atropine (0.1 μM, 20 min equilibration). Fig-

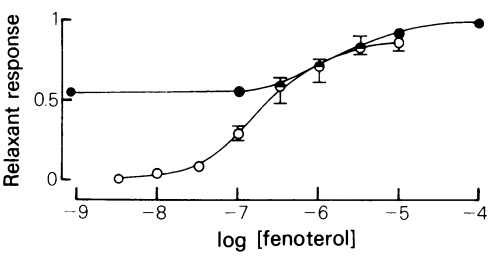

Figure 1 Mean log concentration-effect curves for the relaxant effects of fenoterol in guinea-pig isolated tracheal preparations, in the absence (○) and presence (●) of 0.6 mM Ro 03-7894. Relaxant responses are expressed as a fraction of the carbachol-induced increase in tone observed in the control period. Points show mean ± s.e.mean values (vertical bars) from 4 preparations. For coincident points, assymmetric vertical bars refer to the appropriate half symbol.

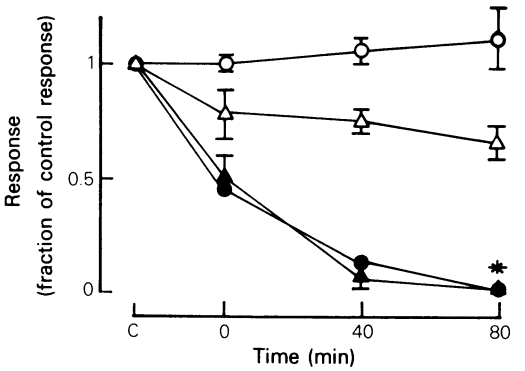

Figure 2 Mean time-dependent changes in contractile responses of tracheal preparations to 0.5 μM carbachol (circles) and 33 mM calcium (triangles). Open symbols are mean results ($n = 4$) from control experiments (no Ro 03-7894), closed symbols represent mean responses ($n = 4$) obtained at the end of a 30 min incubation with 0.6 mM Ro 03-7894 (0 min) and 40 min (3 washes) and 80 min (6 washes) after washout. The asterisk represents mean contraction height ($n = 4$) produced by 5 μM carbachol 80 min after washout of Ro 03-7894. Responses are expressed as a fraction of contraction height observed in the control period (C) of each experiment. Points show mean values and vertical bars (when greater than size of symbol) represent the s.e.mean.

ure 2 shows that Ro 03-7894 produced reductions in the size of the contractions to calcium (33 mM) which were of similar magnitude and time course to those observed with carbachol. Figure 2 also shows that the contractile responses to carbachol and calcium were well maintained when incubation with Ro 03-7894 was omitted.

Uterine smooth muscle

The cumulative addition of (−)-isoprenaline completely relaxed the tone induced by exposure of uterine preparations to a K^+ -depolarizing solution. The mean (±s.e.mean) increase in tone produced by K^+ was 2.4 ± 0.5g ($n = 12$) and the (−)-isoprenaline pD_2 value for relaxant effects was 7.63 ± 0.14 (mean ± s.e.mean, $n = 12$).

The size of the K^+-induced contraction obtained after 30 min equilibration with 0.6 mM Ro 03-7894 was identical to that observed in the immediate control period (ratio K^+ contraction plus Ro 03-7894: K^+ control = 1.04 ± 0.19, mean ± s.e.mean, $n = 4$). Figure 3 shows mean cumulative concentration-effect curves to (−)-isoprenaline in the absence and presence of 0.6 mM Ro 03-7894. The rightward displacement of the (−)-isoprenaline curve was not parallel. The approximate dose-ratios for (−)-isoprenaline at 0.3, 0.5 and 0.8 of the maximal relaxation were 6, 19 and 73, respectively.

In another series of four experiments, curves to (−)-isoprenaline were established 40 min and 80 min after washout of Ro 03-7894. Forty min after washout, the heights of the K^+ -induced contractile responses relative to control were variable, the heights being 0.26, 0.73, 1.78 and 1.74 of control responses. (−)-Isoprenaline completely relaxed all uterine preparations and the mean dose-ratio calculated using concentrations of (−)-isoprenaline which relaxed the tissue to the same absolute tension (corresponding to 0.8 ordinate in Figure 3) was 3.2 ± 1.4 (mean ± s.e.mean, $n = 3$) thus indicating significant reversal of the effect of Ro 03-7894. Responses to K^+ were markedly depressed 80 min after washout. In two preparations there was no response to K^+ whilst in the other two, responses were reduced from 1.78 and 1.74 to 0.5 and 0.6 of the pre-Ro 03-7894 contractile response heights, respectively.

Similar depressant effects were observed when the effect of Ro 03-7894 was investigated on responses to the cumulative addition of calcium in tissues bathed in a Ca^{2+}-free, K^+-depolarizing solution. In tissues which were not treated with Ro 03-7994, responses to K^+ and Ca^+ were well maintained at all times.

Ileal smooth muscle

(−)-Isoprenaline produced a maximal decrease of 59.2 ± 3.8% (mean ± s.e.mean, $n = 8$) in the size of ileal contractions. The pD_2 value for (−)-isoprenaline was 7.35 ± 0.09 (mean ± s.e.mean, $n = 8$). Following the addition of 0.6 mM Ro 03-7894, contractions were completely abolished within 2 min but subsequently recovered over the next 15–20 min, to 10%, 69% and 73% of the pre-drug contraction heights in 3 of the 4 preparations. In the remaining preparation, contractions remained completely

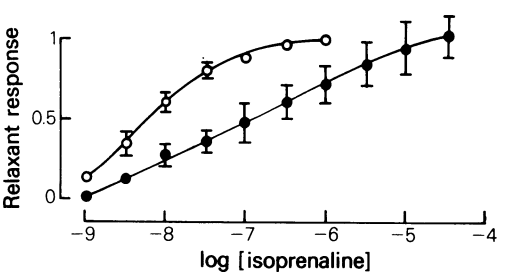

Figure 3 Mean concentration-effect curves for the relaxant effects of (−)-isoprenaline in K^+ -depolarized guinea-pig uterine preparations in the absence (○) and presence (●) of 0.6 mM Ro 03-7894. Relaxant responses are expressed as a fraction of the K^+ -induced contracture observed in the control period. Points show mean values, and vertical bars (if greater than size of symbol) represent s.e.mean, from 4 preparations.

abolished. Superimposed (−)-isoprenaline curves were shifted to the right in the two preparations which exhibited substantial recovery. The dose-ratios for (−)-isoprenaline calculated using concentrations which relaxed the tissue to the same absolute tension, were 59 and 65. There was no evidence of a reduction in the maximal responses to (−)-isoprenaline. In these two preparations, the sizes of the transmurally elicited contractions were progressively depressed to approximately 36% and 23% of control levels 40 min and 120 min after washout, respectively. Due to the low tone it was not possible to re-establish further curves to (−)-isoprenaline.

Contractile responses to electrical stimulation and the relaxant effects of (−)-isoprenaline were unaltered in tissues which were not exposed to Ro 03-7894.

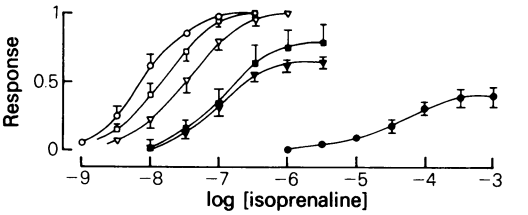

Figure 4 Mean concentration-effect curves for positive inotropic actions of (−)-isoprenaline in electrically stimulated left atrial preparations under control conditions (open symbols) and after treatment (closed symbols) with 0.6 mM Ro 03-7894 for 30 min (○ no washout, □ 60 min washout, ▽ 120 min washout). Responses are expressed as a fraction of the maximal effects to (−) -isoprenaline in the control period. Shown are mean values ($n = 4$) together with vertical bars (if greater than the size of the symbol) representing the s.e.mean.

Atrial muscle

(−)-Isoprenaline produced an increase in the force of contraction of electrically stimulated left atrial preparations with a mean pD_2 value of 7.92 ± 0.08 (mean ± s.e.mean, $n = 16$). The maximal increase in force produced by (−)-isoprenaline represented a 7.01 ± 1.12 fold (mean ± s.e.mean, $n = 16$) increase above resting values. In four experiments concentration-effect curves to (−)-isoprenaline were constructed in the absence and in the presence (30 min after incubation) of various concentrations of Ro 03-7894 (0.001, 0.01, 0.1 and 0.6 mM). Table 1 shows the mean dose-ratios for (−)-isoprenaline calculated from the EC_{50} values of each curve, and the maximal response to the catecholamine expressed as a fraction of that observed in the control period. Basal tension was reduced by $23 \pm 8\%$ (mean ± s.e.mean, $n = 4$) and $52 \pm 7\%$ mean ± s.e.mean $n = 4$) 30 min after incubation with 0.1 mM and 0.6 M Ro 03-7894, respectively. Ro 03-7894 produced a shift to the right of the dose-response curves and depressed the maximal response to (−)-isoprenaline at 0.1 and 0.6 mM. Analysis of the (−)-isoprenaline dose-ratios indicated that the antagonism was not of the competitive type (slope log (dose-ratio − 1) versus log (molar conc. Ro 03-7894) $= 1.46 \pm 0.04$ (mean ± s.e.mean, $n = 4$)).

Table 1 Rightward displacement and depression of maximal positive inotropic effects of (−)-isoprenaline produced by four concentrations of Ro 03-7894 in guinea-pig left atria

Concentration Ro 03-7894 (mM)	*Dose-ratio*	*Fraction control max. response*
0.001	1.6 ± 0.1	0.99 ± 0.02
0.010	5.7 ± 1.0	0.94 ± 0.03
0.100	1761 ± 290	0.78 ± 0.04
0.600	4935 ± 1629	0.40 ± 0.07

The values shown are the mean (−)-isoprenaline dose-ratios (EC_{50} treated: EC_{50} control) and maximal responses to (−)-isoprenaline expressed as a fraction of control curves. Values are means ± s.e.mean from 4 experiments.

Figure 4 shows mean concentration-effect curves to (−)-isoprenaline in the absence and presence of Ro 03-7894 (0.6 mM) and following washout after 60 or 120 min. Sixty and 120 min after washout, the (−)-isoprenaline dose-ratios for half-maximal effects were 12.6 ± 3.9 and 3.5 ± 0.3 (means ± s.e.mean, $n = 4$), respectively, with maximal responses corresponding to 0.79 ± 0.13 and 0.64 0.03% (means ± s.e.mean $n = 4$) of control, respectively. Basal tension remained reduced, $44 \pm 5\%$ of control values (mean ± s.e.mean, $n = 4$) after 120 min washout. In atrial preparations in which Ro 03-7894 was omitted, there was no change in the position, or the maximum responses to isoprenaline over this time period (data not shown).

The cumulative addition of calcium chloride elicited positive inotropic responses in left atrial preparations which were unaffected by the presence of 0.5 μM propranolol. At a concentration of 10 mM, calcium elicited a similar increase in tension to that of (−)-isoprenaline (8.67 ± 0.61 fold, mean ± s.e.mean $n = 8$). After 30 min equilibration with 0.6 mM Ro 03-7894, the response to calcium was reduced to 0.64 ± 0.07 (mean ± s.e.mean $n = 4$) of control, and remained at this level 60 and 120 min after washout (0.65 ± 0.11, 0.62 ± 0.10 (means ± s.e.mean, $n = 4$), respectively). In studies in which Ro 03-7894 was omitted, the responses to calcium were unaltered (data not shown).

Cumulative concentration-effect curves for the positive inotropic actions of histamine (in presence of propranolol (μM) elicited a mean maximal increase in tension of 2.34 ± 0.36 fold (mean ± s.e.mean, $n = 8$). Following treatment of atria with 0.6 mM Ro 03-7894 and 120 min washout, responses to histamine could be elicited in only 1 of the 4 preparations. In the responsive preparation, the maximal response to histamine was reduced to 0.56 of the control level. In untreated tissues the maximal increase in tension to histamine 120 min after washout was 5.06 ± 1.65 fold (mean ± s.e.mean, $n = 4$).

Effective refractory period in the absence, presence and after washout of Ro 03-7894 was assessed in 4 experiments. In the presence of 0.6 mM Ro 03-7894, the refractory period was increased to 130 ± 4% (mean ± s.e.mean $n = 4$) of control (92 ± 5ms, mean ± s.e.mean, $n = 4$). This effect of Ro 03-7894 was readily reversed by washing for 40 min (98 ± 2%, mean ± s.e.mean, $n = 4$), and furthermore, remained unaffected after washing for a total of 80 min (99 ± 4%, mean ± s.e.mean, $n = 4$) and 120 min (95 ± 6%, mean ± s.e.mean, $n = 4$).

Table 2 shows the ability of [^{125}I]-CYP to bind to treated and untreated atrial muscle membranes. [^{125}I]-CYP bound with high affinity to membranes prepared from either atrial treatment groups. Scatchard plots were linear indicating that [^{125}I]-CYP was binding to a homogeneous population of binding sites and Hill coefficients were not significantly different from unity, indicating an absence of co-operativity in binding. There was no difference in the K_D values or the maximal density of binding sites (B_{max}) between the treated and control membranes.

As a further check, the ratio of the B_{max} values (treated: control) was also calculated. The mean value of 1.28 ± 0.11 (mean ± s.e.mean, $n = 3$) indicated that treatment with Ro 03-7894 did not reduce the maximum density of binding sites.

Table 2 Dissociation constant (K_D), maximal number of binding sites (B_{max}) and Hill coefficient for (−)-[^{125}I]-iodocyanopindolol binding to control (no Ro 03-7894 plus 120 min washout) and treated (0.6 mM Ro 03-7894 followed by 120 min washout) guinea-pig left atrial membranes

	K_D (pmol l^{-1})	B_{max} (pmol g^{-1} wet wt)	*Hill coefficient*
Control ($n = 3$)	49 ± 7	1.06 ± 0.32	0.97 ± 0.02
Treated ($n = 3$)	62 ± 16	1.29 ± 0.25	0.95 ± 0.04

Values shown are mean ± s.e.mean from 3 paired experiments (see Methods for more details).

Discussion

In isolated left atrial preparations Ro 03-7894 produced a marked rightward shift of the positive inotropic concentration-effect curves to (−)-isoprenaline. The antagonism was not of a competitive type since the value of slope was significantly greater than unity. However, it is not possible to attribute this rightward shift to antagonism at β_1-adrenoceptor sites, since a considerably smaller shift was observed in ileal preparations which also contain a homogeneous population of this β-receptor subtype (Williams & Broadley, 1982). Thus the actions of Ro 03-7894 on responses to (−)-isoprenaline are relatively specific for cardiac as opposed to smooth muscle, i.e. not related to any direct action (that it may have) on β-receptors.

At a concentration of 0.6 mM, Ro 03-7894 was devoid of any marked β_2-receptor antagonistic action against the relaxant effects of fenoterol in tracheal and (−)-isoprenaline in uterine preparations. In the latter tissue there was a non-parallel rightward displacement of the (−)-isoprenaline dose-response curves which was readily reversed by washing the tissue. In neither preparation was there evidence of an irreversible action since there was no reduction in the maximal response to the agonists, either in the presence or on subsequent washout of Ro 03-7894. On the basis of these results, Ro 03-7894 would not be a suitable tool for investigating receptor mechanisms at β_2-adrenoceptor sites.

Ro 03-7894 depressed contractile responses of ileal, tracheal and uterine preparations. The most striking feature was that the depression of responses progressively increased with time after washout of the drug. The depression of the contractile responses in these preparations was not due to a time-dependent deterioration in responsiveness since contractions were well maintained over a similar time-course in experiments in which Ro 03-7894 was not present. These results indicate that Ro 03-7894 produces a non-specific depressant effect on smooth muscle which is unaffected by washout of the drug.

In left atrial preparations, the marked rightward shift of (−)-isoprenaline dose-response curves was essentially reversed by washing for 60 or 120 min, while the maximum responses and the basal tension remained depressed. However, unlike tracheal, ileal and uterine preparations, the basal tension did not show a marked time-dependent enhancement of this depressant effect. The lack of a time-dependent inhibition may reflect a difference in the mechanism of action of Ro 03-7894 in atrial as opposed to smooth muscle or merely reflect differences between the two types of muscle.

In contrast to prevous findings (Nicholson & Broadley, 1978), the results of the present study

indicate that the inhibition of atrial responses was not specific for β-receptor mediated responses, since the positive inotropic effects of calcium, histamine and (−)-isoprenaline were inhibited by Ro 03-7894 even 120 min after washout. Furthermore, with 0.6 mM of the compound, responses to the three agonists were depressed to a similar degree. For all three agonists, there was no inhibition of responses in left atrial preparations which had not been treated with Ro 03-7894. Thus, these results cast doubt on the classification of Ro 03-7894 as an irreversible β-adrenoceptor antagonist. Similar findings to those of the present study have recently been reported by Baker & Posner (1983) in isolated left atria from rats.

While there are obvious differences in the time-course and extent of the depressant effects of Ro 03-7894 in the four tissues (atrium, uterus, ileum, trachea), the overall effects of this drug suggested a common non-specific type of interaction which was initiated during the 30 min equilibration period and which continued after washout. Such a drug effect has previously been described for the antagonism of calcium responses by intracellular actions of cinnarizine, pimozide and fendilene in guinea-pig taenia preparations (Spedding, 1982; 1983). It is of interest that the results from the effective refractory period experiments do not provide an explanation for the continued inhibitory effects after washout of the drug.

Although Ro 03-7894 has been shown to possess a non-specific depressant effect, this does not exclude the possibility that it may behave as an irreversible antagonist at β-adrenoceptor sites. In one previous study (Rankin & Broadley, 1982) it was shown that the density of [^{3}H]-dihydroalprenolol binding sites in guinea-pig ventricle membrane preparations, remained reduced after incubation and removal of Ro 03-7894 by a washing procedure which effectively removed bound propranolol. The radioligand chosen for the present study was [^{125}I]-CYP (Hoyer *et al.*, 1982), which due to its high affinity for the β-adrenoceptor and also its high intrinsic specific activity, could be used to examine binding in left atrial tissue.

In order to assess as accurately as possible the effects of Ro 03-7894 on the maximal density of binding sites, the atrial halves were treated exactly as for the (−)-isoprenaline – Ro 03-7894 inotropic studies, except that dose-response curves to (−)-isoprenaline were omitted. In addition, the binding assay buffer used was a Krebs-phosphate buffer which was identical to the Krebs-bicarbonate solution except that the bicarbonate was exchanged for phosphate salts, so as to maintain a pH of 7.4 in the absence of aeration with carbogen. When assayed under these conditions, it was apparent that Ro 03-7894 did not reduce the maximal density of binding sites at times when responses to (−)-isoprenaline were depressed by approximately 40% in the inotropic studies. Thus, these results cast doubt on the ability of Ro 03-7894 to form an irreversible bond with the β-adrenoceptor. The present results are in accord with those of Baker & Posner (1983) who examined the effects of Ro 03-7894 on [^{3}H]-dihydroalprenol binding in rat ventricular and lung membrane preparations.

The radioligand binding techniques used in the present study and those used by Rankin & Broadley (1982) are markedly different, and may well account for the diverse results. In particular, it should be noted that in the present study, the washing procedure was more extensive and was performed on intact atria as in the corresponding organ bath studies, while Rankin & Broadley (1982) used a limited washing procedure of membranes prepared from ventricle muscle. In addition, conditions for an accurate comparison as to the reversibility of binding by propranolol and Ro 03-7894 were not identical in the study by Rankin & Broadley (1982), since the concentration of propranolol used was 1/10th of the IC_{50} value, whilst a concentration equal to the IC_{50} was used for Ro 03-7894.

In conclusion, the results indicate that Ro 03-7894 is not a suitable tool for investigating mechanisms at β-adrenoceptor sites since it possesses a non-specific inhibitory effect on isolated smooth and cardiac muscle preparations. As there is no evidence for an Ro 03-7894-induced reduction in the maximal density of [^{125}I]-CYP binding sites, it appears that the compound is not an irreversible antagonist. Its apparent 'irreversible' activity (i.e. depression of responses to (−)-isoprenaline in electrically stimulated left atrial preparations following washout of Ro 03-7894 from the tissue) is more likely to be a reflection of its non-specific inhibitory effect. On the basis of the present results and those of Baker & Posner (1983) it is suggested that due care should be exercised in interpreting the actions of this drug on the basis of irreversible β-adrenoceptor blockade.

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