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COMPARISON OF THE APPARENT IRREVERSIBLE β-ADRENOCEPTOR ANTAGONIST Ro 03-7894 WITH PROPRANOLOL IN CARDIAC VENTRICULAR MUSCLE BY PHARMACOLOGICAL AND RADIOLIGAND BINDING TECHNIQUES

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Abstract—Membrane fractions were prepared from guinea-pig ventricular muscle and the specific binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) assessed. The dissociation constant (K_i) of (±)-propranolol was determined (6.9 nM) from its ability to displace [^{3}H]DHA binding. This compared with the pA_2 value of propranolol of 8.32 (dissociation constant, 4.8 nM) determined for the antagonism of isoprenaline-induced positive inotropic responses of papillary muscles from guinea-pig hearts. Scatchard analysis of the saturation curves for specific [^{3}H]DHA binding showed that in the presence of propranolol, the displacement was characteristic of competitive antagonism. That is, there was no change in the total β-adrenoceptor binding sites (B_{max}) but an apparent reduction of the dissociation constant (K_D) of [^{3}H]DHA. This antagonism was fully reversed by washing membranes that had been previously incubated with propranolol. In contrast, in the presence of the β-adrenoceptor antagonist, Ro 03-7894, the Scatchard plots were displaced in a manner characteristic of irreversible antagonism. The B_{max} was significantly reduced. This antagonism was resistant to washout, with the Scatchard plots still showing a reduced B_{max} and no change in the dissociation constant (K_D) of [^{3}H]DHA. This apparent irreversible antagonism by Ro 03-7894 was also demonstrated in guinea-pig isolated papillary muscles. The maximum of the dose–response curve to isoprenaline, constructed after incubation with Ro 03-7894 and a 3 hr bath-washout, was depressed by 89.5 ± 7.5%.

Interactions of agonists and antagonists with β-adrenoceptors can be examined pharmacologically in isolated tissues and from the binding of radiolabelled ligands such as (−)-[^{3}H]dihydroalprenolol ([^{3}H]DHA) [1] to tissue membrane fractions. Radioligand binding techniques can be employed to distinguish between competitive and irreversible antagonists. These antagonists modify the saturation curves for the binding of increasing concentrations of the radioligand differently and produce different effects upon the Scatchard analysis of these data. The differences between phentolamine (competitive) and phenoxybenzamine (irreversible) at α-adrenoceptors have been successfully demonstrated in this way [2]. However, irreversible β-adrenoceptor antagonists have only recently been described. Chloropractolol was claimed to have irreversible antagonist activity from pharmacological data [3], but this was soon discounted and attributed merely to its indirect sympathomimetic activity leading to the erroneous conclusion that it was an irreversible β-adrenoceptor antagonist [4]. Subsequently, two further antagonists, FM 24 [5–8] and NHNP-NBE [9, 10] have also been shown to behave irreversibly by the use of radioligand binding studies. The irreversible activity of the latter of these compounds has also been demonstrated in cat isolated papillary muscles [10]. In these laboratories, the compound Ro 03-7884 has been shown to irreversibly antagonize the β-adrenoceptor-mediated positived inotropic and chronotropic responses of guinea-pig isolated atria [11, 12]. Furthermore, the antagonism of cardiac β-adrenoceptors by Ro 03-7894 *in vivo* is prolonged [13].

The present study was undertaken to examine whether this profile of irreversible antagonism by Ro 03-7894 was obtained by the application of radioligand binding of [^{3}H]DHA to membrane fractions of guinea-pig ventricular muscle. Throughout the study Ro 03-7894 was compared with propranolol as the reference competitive β-adrenoceptor antagonist. The effects of both antagonists upon [^{3}H]DHA binding were also compared with their activities upon the responses of guinea-pig isolated papillary muscle to isoprenaline. Thus radioligand binding and pharmacological activity in ventricular muscle could be compared directly.

MATERIALS AND METHODS

Preparation of ventricular muscle membranes. Guinea-pigs of either sex and weight range 300–500 g were killed by a blow on the head and exsanguinated. The thorax was opened and the heart removed. The ventricles were placed in 10 vol. of ice-cold buffer (0.25 M sucrose, 5 mM Tris (hydroxymethyl) methylamine, 1 mM magnesium chloride, pH 7.5) and all subsequent procedures carried out at 4°. The

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tissue was homogenized using an Ultraturex homogenizer (15 second burst) followed by 3 strokes with a motor-driven Teflon pestle homogenizer.

The homogenate was filtered through muslin and centrifuged for 10 min at 1000 *g* to remove connective tissue, unbroken cells and cell debris. The supernatant was then centrifuged for 10 min at 30,000 *g* and the pellet resuspended in buffer (20 mM potassium orthophosphate, 1 mM magnesium chloride, pH 7.5). This washing process was repeated a further two times before the final pellet was resuspended in assay buffer (50 mM potassium orthophosphate, 4 mM magnesium chloride, pH 7.5).

Binding of [^{3}H]*DHA.* Each test tube for the binding assay contained 0.2–0.8 mg of membrane protein assayed by the method of Lowry *et al.* [14] in a 100 μl sample; [^{3}H]DHA (42–102 Ci mmole^{-1}, 2 nM) in a 50 μl sample; and when appropriate, 50 μl of propranolol or Ro 03-7894. The final volume was made up to 250 μl with assay buffer. The antagonists, propranolol and Ro 03-7894, were preincubated with the membrane protein and buffer for 10 min at 38° before addition of the [^{3}H]DHA. The assay tubes were incubated for 20 min at 38°, at the end of which the reaction was terminated by addition of 1 ml of ice-cold assay buffer followed by immediate filtration through Whatman GF/B glass fibre filters supported on a 12-port filter manifold (Millipore). The filters were washed with 3 × 5 ml of ice-cold assay buffer.

The filters were dried in an oven at 60° for 2 hr prior to adding 3.5 ml of scintillation fluid comprising 12.05 mM 2,5-diphenyloxazole and 0.017 mM dimethylPOPOP in a 2:1 mixture of toluene and Triton X-100. Membrane-bound [^{3}H]DHA trapped in the filters was counted in a Beckman LS-235 liquid scintillation counter. In each experiment, non-specifically bound [^{3}H]DHA was determined by incubating membrane protein and [^{3}H]DHA with 200 μM (−)-isoprenaline. Specific binding was thus obtained by deducting this value from the total binding of [^{3}H]DHA for each sample.

Washout experiments. In order to try and wash the antagonists from the membrane binding sites, the method was modified so that membrane samples containing 2.6–10.6 mg of membrane protein in 1.3 ml of assay buffer were incubated with 650 μl of propranolol, Ro 03-7894 or assay buffer in a 50 ml plastic centrifuge tube for 30 min at 38°. The ratio of membrane to drug was therefore the same as in non-washout experiments. These samples were then centrifuged for 10 min at 30,000 *g* and resuspended in 1.3 ml assay buffer with shaking for 30 min to wash. After re-centrifugation and final resuspension in 1.3 ml of assay buffer, 100 μl membrane protein samples were incubated with [^{3}H]DHA as described above.

Papillary muscle preparations. Left ventricular papillary muscles were rapidly dissected from the guinea-pig hearts prior to their use for the binding assay. They were placed in Krebs–bicarbonate solution of the following composition in mM: NaCl 118.5; glucose 11.7; $NaHCO_3$ 25; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; KCl 4.7; $CaCl_2 \cdot 2H_20$ 1.9 gassed with 5% CO_2 in oxygen. Papillary muscle preparations were mounted on perspex tissue holders, secured by the chordae tendineae, and passing through bipolar coil electrodes. They were placed in 50 ml organ baths filled with Krebs–bicarbonate solution gassed with 5% CO_2 in oxygen and maintained at 38°. The muscles were attached by cottons secured at their proximal ends to isometric transducers (Dynamometer, UF1, 57 g sensitivity range) and placed under 0.7–1.0 g resting diastolic tension. They were paced electrically with field stimulation at 2 Hz with square-wave pulses of 5 msec duration and voltage of 50% above threshold delivered by an SRI stimulator (type 6053). Isometric tension was recorded on a Devices M19 polygraph.

After an initial equilibration period of 30 min, during which several changes of bathing were made, a dose–response curve to isoprenaline was obtained by cumulative addition in half logarithmic increments in concentration.

pA_2 Determination. The antagonism of isoprenaline by propranolol was assessed by determination of the pA_2 value using the method of Arunlakshana and Schild [15]. After the initial dose–response curve to isoprenaline and return of the developed tension to the pre-isoprenaline resting level, propranolol (10^{-8} M) was incubated with the tissue for 30 min before constructing a further dose–response curve to isoprenaline in its presence. The isoprenaline was washed from the bath with Krebs–bicarbonate solution containing propranolol (10^{-7} M) and when the pre-isoprenaline resting developed tension was restored, a 30 min incubation period was commenced. The next dose–response curve to isoprenaline was then constructed. This procedure was then repeated using 10^{-6} M propranolol. Control experiments were then performed to allow for changes in sensitivity occurring during the course of the pA_2 determination. In these, four dose–response curves to isoprenaline were obtained without the intervention of propranolol.

Responses were then measured as the increase in tension above the resting developed tension and dose–response curves plotted as a percentage of the maximum increase for each curve. EC_{50} values were determined and the shift of the dose–response curve produced by each concentration of propranolol was expressed as the dose-ratio (DR). These were corrected by dividing by the mean ($n = 4$) correction DR from control experiments. Values of log (DR − 1) were plotted against molar concentration of propranolol and the calculated regression line drawn, the intercept on the abscissa yielding the pA_2 value.

Antagonism of isoprenaline by Ro 03-7894. After an initial dose–response curve to isoprenaline, the papillary muscles were washed and allowed to return to the pre-isoprenaline resting level before incubating with Ro 03-7894 (3.26×10^{-4} M) for 30 min. This was followed by nine washes, at 20 min intervals, with fresh bathing medium before a final dose–response curve to isoprenaline was obtained. Control experiments were performed in an identical manner except that the tissues were incubated with Krebs–bicarbonate solution instead of Ro 03-7894. Responses were measured as the increase in tension above the resting developed tension. The change in mean ($n = 4$) response size occurring between first and second dose–response curves of control experi-

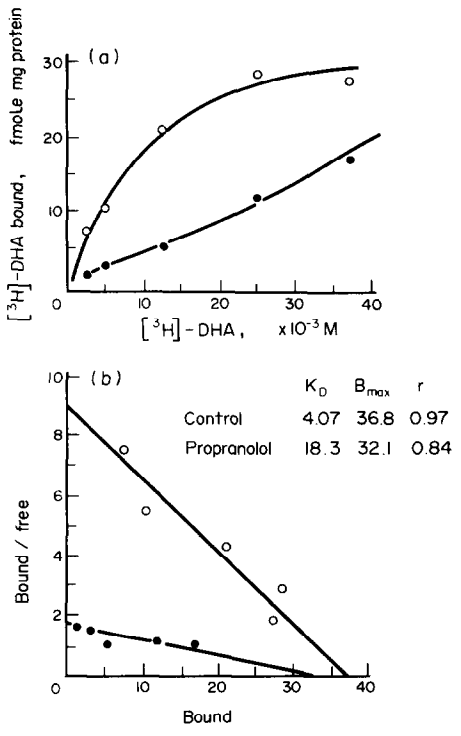

Fig. 1. Effect of propranolol upon specific binding of [³H]DHA to guinea-pig ventricular membranes. (A) Saturation curves for specific binding of increasing concentrations of [³H]DHA to guinea-pig ventricular membranes in the absence (○) and presence (●) of propranolol (6.8×10^{-10} M) at 38°. (B) Scatchard analysis of the saturation curves in A; the ratio of bound (fmole mg^{-1} protein) to free (nM) [³H]DHA is plotted against bound ligand. These are representative curves from one of 5 experiments performed in duplicate, from which the K_D (nM), B_{max} (fmole mg $protein^{-1}$) and regression coefficient (r) values are shown.

ments at each concentration of isoprenaline was expressed as a fraction. These factors were then applied to individual responses to the corresponding concentration of isoprenaline before Ro 03-7894 in test experiments. The mean ($n = 4$) corrected increases in tension were then plotted as a percentage of the maximum increase (corrected) before Ro 03-7894.

Drugs. Ro 03-7894 [1(5-acetylaminobenzofuran-2-yl)-hydroxy-2-isopropylaminoethane] was synthesized and kindly donated by Roche Products Ltd. (Welwyn Garden City, U.K.). (±)-Propranolol hydrochloride was kindly donated by ICI Pharmaceuticals, U.K. and (−)-isoprenaline bitartrate dihydrate by Ward Blenkinsop Ltd., U.K.

(−)-[4,6,propyl-³H]Dihydroalprenolol (42–102 Ci $mmole^{-1}$) was obtained from the Radiochemical Centre (Amersham, U.K.). All other chemicals were of analytical grade.

RESULTS

Displacement of [³H]*DHA binding by propranolol.* Specific binding of [³H]DHA (2.0 nM) to cardiac membranes was determined in the presence of increasing concentrations of propranolol. The IC_{50} (concn to produce 50% displacement) obtained from the mean ($n = 4$) displacement curve was 6.5 nM. This yielded a dissociation constant (K_i) for propranolol of 6.9 nM. This was calculated from the equation $k_i = IC_{50/1} + S/K_D$ [10], where S is the concentration of [³H]DHA and K_D is the dissociation constant of the binding ligand (4.9 nM) which was obtained from subsequent experiments (Table 1).

Effect of propranolol on [³H]*DHA binding.* Saturation curves for the specific binding of increasing concentrations of [³H]DHA were obtained in the absence and presence of propranolol (6.8×10^{-10} M) (Fig. 1A). These curves were subjected to Scatchard analysis by replotting the ratio of bound to free against bound ligand (Fig. 1B). This yielded the equilibrium dissociation constant (K_D) for specific [³H]DHA binding from the negative reciprocal of the slope, and the total number of binding sites (B_{max}) from the intercept on the abscissa. In the absence of propranolol, the mean K_D value for [³H]DHA binding was 4.9 ± 1.1 nM. In the presence

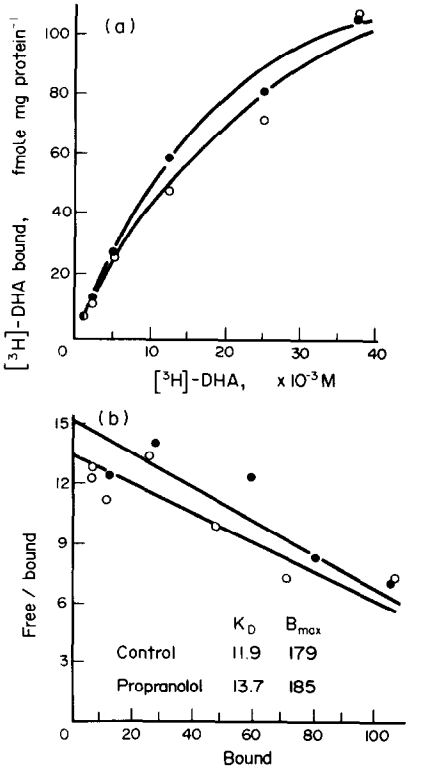

Fig. 2. Effect of incubating guinea-pig ventricular membranes with propranolol and subsequent washout upon specific [³H]DHA binding. (A) Saturation curves for specific binding of increasing concentrations of [³H]DHA to guinea-pig ventricular membranes exposed to propranolol (6.8×10^{-10} M) for 10 min at 38° and then washed twice (●). Control membranes (○) were treated identically, but were not exposed to propranolol. (B) Scatchard analysis of the saturation curves in A; the ratio of bound (fmole mg $protein^{-1}$) to free (nM) [³H]DHA is plotted against bound ligand. These are representative curves from one of 4 experiments performed in duplicate, from which the K_D (nM) and B_{max} (fmole mg $protein^{-1}$) values are shown.

Table 1. Effects of propranolol and Ro 03-7894 upon dissociation constants and total number of β-adrenoceptor binding sites of [H³]DHA binding to guinea-pig ventricular membranes

Antagonist/treatment	K_D (nM)	Mean (test–control)	B_{max} (fmole mg protein^{-1})	Mean (test–control)	n
Propranolol, in presence of (6.8×10^{-10} M)	17.8 ± 2.0†	12.9 ± 1.9	78.7 ± 18.8	−3.8 ± 11.6	5
Control	4.9 ± 1.1		82.4 ± 15.1		5
Propranolol, after washout (6.8 ± 10^{-10} M)	13.1 ± 2.7	0.92 ± 1.4	93.6 ± 37.4	−0.8 ± 5.4	4
Control	12.2 ± 2.8		94.4 ± 38.6		4
Ro 03-7894, in presence of (6.5×10^{-8} M)	1.8 ± 0.3†	−5.6 ± 0.8§	24.2 ± 5.3†	−111.8 ± 19.9§	5
Control	7.4 ± 0.9		136.1 ± 17.6		5
Ro 03-7894, after washout (6.5×10^{-8} M)	10.9 ± 1.7	3.5 ± 5.4	46.6 ± 18.4*	−82.4 ± 24.9‡	4
Control	7.2 ± 3.5		134.1 ± 27.5		4

Mean (± S.E.M.) dissociation constants (K_D) and total number of binding sites (B_{max}) were determined from Scatchard plots of individual experiments performed in duplicate. Specific binding of [³H]DHA was determined in the presence of propranolol or Ro 03-7894 or after their incubation with the membranes followed by washout. Controls were performed identically to the corresponding test experiment except that the antagonist was omitted. Mean differences between individual antagonist-incubated membranes and their corresponding controls for K_D and B_{max} values were also calculated.

Statistical analysis by two-tailed Student's t-test was performed to compare: (a) mean test K_D and B_{max} values with their corresponding controls, significance levels for differences are depicted by *$P < 0.05$ and †$P < 0.001$, and (b) the mean (test–control) values for propranolol and Ro 03-7894, significance levels are depicted by ‡$P < 0.05$ and § $P < 0.001$. Analysis by the non-parametric Mann–Whitney U-test showed the same differences to be significant at $P < 0.05$.

of propranolol, there was a significant ($P < 0.001$) increase in the mean ($n = 5$) apparent K_D value, but no change in B_{max} (Table 1).

The cardiac membranes were then preincubated with propranolol (6.8×10^{-10} M) and then washed before obtaining saturation curves for [³H]DHA binding. The control curves for membranes exposed to the washing procedure only, showed no significant difference ($P > 0.05$) from the control curves that had received no washing, with respect to K_D and B_{max} values (Table 1). The saturation curves and Scatchard plots for membranes incubated with propranolol before washing were virtually superimposed upon those for control washed membranes (Fig. 2A and B). The mean ($n = 4$) K_D and B_{max} values of 13.1 ± 2.7 nM and 93.6 ± 37.4 fmole mg protein^{-1} for propranolol-incubated membranes were not significantly different ($P > 0.05$) from the values of 12.2 ± 2.8 and 94.4 ± 38.6 respectively in control membranes (Table 1).

Effect of Ro 03-7894 on [³H]DHA binding. Saturation curves for the specific binding of [³H]DHA in increasing concentrations were obtained in the absence and presence of Ro 03-7894 (6.5×10^{-8} M) (Fig. 3A). This concentration was the IC_{50} for displacement of [³H]DHA binding determined in preliminary experiments. The slope of the Scatchard plot in the presence of Ro 03-7894 was steeper than the control plot (Fig. 3B) and this resulted in a significant ($P < 0.001$) reduction in the mean ($n = 5$) K_D values. Also, the total number of binding sites (B_{max}) significantly fell ($P < 0.001$) from the 136.1 ± 17.6 to 24.2 ± 5.3 fmole mg protein^{-1} (Table 1). The differences in individual K_D and B_{max} values between membranes in the presence of Ro 03-7894 and their corresponding controls were calculated (Table 1). The mean differences in K_D (−5.6 ± 0.8 nM) and B_{max} (−111.8 ± 19.9 fmole mg protein^{-1}) values were significantly greater ($P < 0.001$) than the differences obtained in the presence of propranolol (K_D, 12.9 ± 1.9 nM; B_{max}, −3.8 ± 11.6 fmole mg protein^{-1}).

When Ro 03-7894-preincubated membranes were subjected to the washing procedures before obtaining the [³H]DHA saturation curves, there was still a significant ($P < 0.05$) reduction in the mean ($n = 4$) total number of binding sites (B_{max}, 46.6 ± 18.4 fmole mg protein^{-1}) compared with control washed membranes (134.1 ± 27.5 fmole mg protein^{-1}) (Fig. 4). The mean K_D values, however, were not significantly different ($P > 0.05$) at 10.9 ± 1.7 and 7.2 ± 3.5 nM respectively (Table 1). The mean differences between individual B_{max} values and their corresponding controls were calculated and the value obtained after washout of Ro 03-7894 (−82.4 ± 24.9 fmole mg protein^{-1}) was significantly greater ($P < 0.01$) than that occurring after washout of propranolol (−3.8 ± 11.6 fmole mg protein^{-1}).

Effect of propranolol on positive inotropic responses of guinea-pig papillary muscle to isoprenaline. Increasing concentrations of propranolol produced progressive parallel displacement of the dose–response curves to isoprenaline obtained in guinea-pig papillary muscles. The corrected dose-ratios for this displacement from four experiments were plotted as log (DR-1) against concentrations of propranolol (Fig. 5). The intercept on the concentration axis was 4.8 nM which is the equilibrium dissociation constant (K_B) for propranolol. This yields a pA_2 value of 8.32, since the pA_2 is the negative logarithm of the K_B value. The mean pA_2 value calculated from the individual Schild plots of

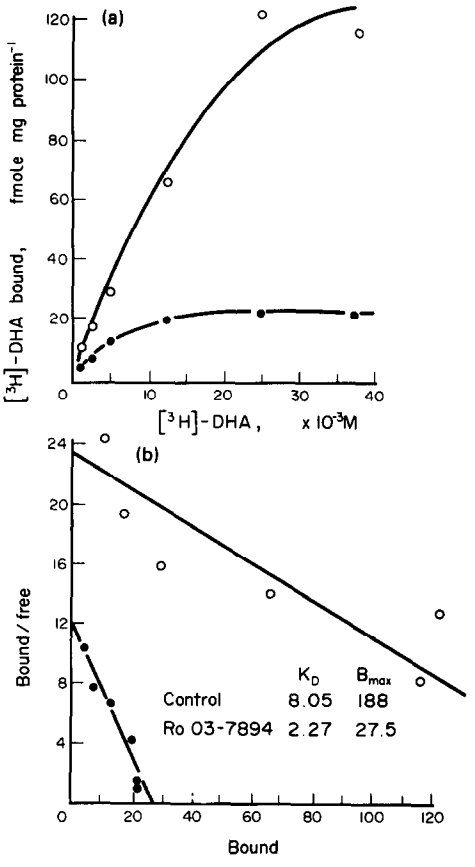

Fig. 3. Effect of the β-adrenoceptor antagonist Ro 03-7894 upon specific binding of [^{3}H]DHA to guinea-pig ventricular membranes. (A) Saturation curves for specific binding of increasing concentrations of [^{3}H]DHA to guinea-pig ventricular membranes in the absence (○) and presence (●) of Ro 03-7894 (6.5×10^{-8} M) at 38°. (B) Scatchard analysis of the saturation curves in A; the ratio of bound (fmole mg protein^{-1}) to free (nM) [^{3}H]DHA is plotted against bound ligand. These are representative curves from one of 5 experiments performed in duplicate, from which the K_D (nM) and B_{max} (fmole mg protein^{-1}) values are shown.

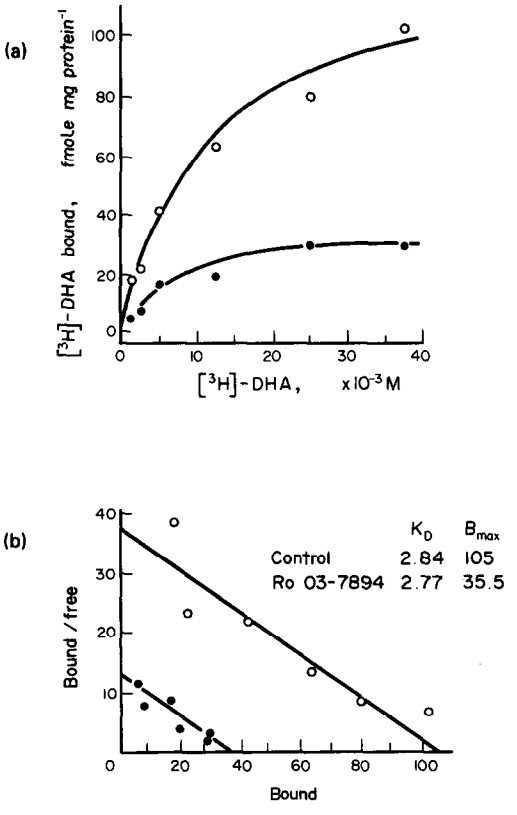

Fig. 4. Effect of incubating guinea-pig ventricular membranes with Ro 03-7894 and subsequent washout upon specific [^{3}H]DHA binding. (A) Saturation curves for specific binding of increasing concentrations of [^{3}H]DHA to guinea-pig ventricular membranes exposed to Ro 03-7894 (6.5×10^{-8} M) for 10 min at 38° and then washed twice (●). Control membranes (○) were treated identically, but were not exposed to Ro 03-7894. (B) Scatchard analysis of the saturation curve in A; the ratio of bound (fmole mg protein^{-1}) to free (nM) [^{3}H]DHA is plotted against bound ligand. These are representative curves from one of 5 experiments performed in duplicate, from which the K_D (nM) and B_{max} (fmole mg protein^{-1}) values are shown.

the four experiments was 8.14 ± 0.25 and the mean slope was 0.94 ± 0.04.

Effect of Ro 03-7894 on the positive inotropic responses of guinea-pig papillary muscle to isoprenaline. Mean ($n = 4$) dose–response curves to isoprenaline were obtained in guinea-pig papillary muscles before and after a 30-min incubation with Ro 03-7894 (3.26×10^{-4} M) followed by washing of the bath for 3 hr (Fig. 6). The dose–response curve was depressed by the incubation with Ro 03-7894, the maximum tension increase being significantly ($P < 0.01$) depressed by 89.5 ± 7.5% even after extensive washing from the bath.

DISCUSSION

The dissociation constant (K_i) for the displacement by (±)-propranolol of specific [^{3}H]DHA binding to cardiac membrane fractions prepared from guinea-pig ventricular muscle was calculated to be 6.9 nM. This value compares with the range of values (2.4–17 nM) found by others for displacement by either (−)- or (±)-propranolol in myocardial membranes of other species [17, 18]. It is also similar to the values obtained by Kaumann [19] for the direct binding of (−)-[^{3}H]propranolol to kitten ventricular membranes. The pA_2 value of (±)-propranolol, determined from the antagonism of isoprenaline-induced positive inotropic responses of guinea-pig isolated papillary muscle, was 8.32. This is equivalent to a dissociation constant of 4.8 nM. This agrees with pA_2 values obtained in guinea-pig left and right atria which range from 8.23 to 8.79 [20–23]. It also compares with the value for (−)-propranolol obtained in papillary muscles of other species, such as the kitten [19]. However, as far as we are aware, there are no previous literature reports of pA_2 values in papillary muscles from guinea-pigs.

The similarity between the dissociation constants for propranolol measured at 38° from β-adrenoceptor

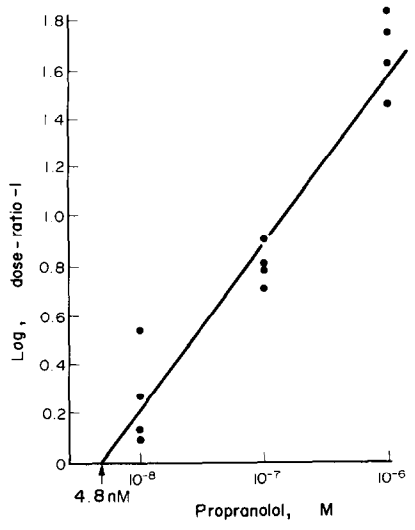

Fig. 5. Arunlakshana and Schild plot for the antagonism of the isoprenaline-induced positive inotropic responses of guinea-pig papillary muscle by propranolol. Dose–response curves for the increase in tension to isoprenaline were obtained before and in the presence of propranolol (10^{-8}, 10^{-7} and 10^{-6} M) with each papillary muscle. The shifts of the dose–response curves by propranolol were expressed as dose-ratios (DR) which were corrected for sensitivity changes from control experiments as described in the text. The corrected dose-ratios from 4 experiments are plotted as log (DR-1) against molar concentration of propranolol (log scale). The calculated regression line (regression coefficient, 0.85) yields a pA_2 value of 8.32 from the intercept on the concentration axis.

binding in ventricular membranes (6.9 nM) and pharmacologically in guinea-pig papillary muscles (4.8 nM) suggests that the β-adrenoceptor binding sites of cardiac membrane fractions—for antagonists at least—did not differ from those in the intact

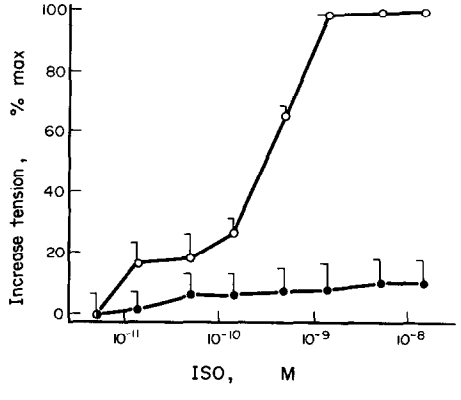

Fig. 6. Effect of Ro 03-7894 upon isoprenaline-induced positive inotropic responses of guinea-pig papillary muscle. Dose–response curves to isoprenaline were obtained before (○) and after the papillary muscles had been incubated with Ro 03-7894 (3.26×10^{-4} M) for 30 min and washed for 3 hr every 20 min (●). Responses were measured as the increases in tension and plotted as a percentage of the pre-Ro 03-7894 maximum increase in tension. The pre-Ro 03-7894 curve was corrected for sensitivity changes from control experiments as described in the text. Each point represents the mean of 4 preparations (± S.E.M.).

papillary muscle. Previous attempts to correlate dissociation constants for β-adrenoceptor antagonists obtained pharmacologically and from binding studies have yielded variable results [17, 18, 21, 24, 25]. However, these comparisons were made between ventricular binding and atrial responses and in some, different species were used [17, 18]. Only Kaumann [19] appears to have previously compared binding in ventricular muscle membranes with papillary muscle responses, both from kitten hearts.

The mean dissociation constant (K_D) of [^{3}H]DHA, measured from Scatchard analysis of the saturation curve for the binding of increasing concentrations of [^{3}H]DHA, was 4.9 ± 1.1 nM. This value compares favourably with the value obtained by others for [^{3}H]DHA binding to guinea-pig ventricular muscle membranes at 37° [26]. In the presence of propranolol, the slope of the Scatchard plot was depressed with a reduction of the apparent K_D for [^{3}H]DHA binding. However, there was no change in the total number of binding sites (B_{max}). This displacement of the Scatchard plot is characteristic of a competitive antagonism for the [^{3}H]DHA binding sites [2]. Furthermore, the antagonism was shown to be reversible by the fact that when membranes exposed to the same concentration of propranolol for the same time were washed twice, the specific binding was restored to the control level, with no difference in apparent dissociation constant or total binding sites. This confirms previous observations of the removal of propranolol from ventricular membranes [5, 10].

The binding of [^{3}H]DHA to cardiac membrane β-adrenoceptors was therefore modified by propranolol in a characteristic manner; competitively and reversibly. This profile thus serves as a reference for comparison with the effect of the β-adrenoceptor antagonist Ro 03-7894. When specific [^{3}H]DHA binding was assessed in the presence of Ro 03-7894, the plateau of the saturation curve was depressed, indicative of a reduction of total available binding sites. The Scatchard analysis yielded a significantly reduced B_{max} value which is characteristic of irreversible antagonism [2]. The apparent dissociation constant (K_D) was also surprisingly reduced, suggesting that there was some increase in the apparent affinity of the [^{3}H]DHA for the β-adrenoceptor binding sites. Ro 03-7894 therefore contrasts with propranolol in that it reduces the total number of specific binding sites. It also differs in that twice washing of the membranes after incubation failed to restore the number of binding sites to the control level of washed membranes not exposed to Ro 03-7894. In this case, the Scatchard plots for control and Ro 03-7894-preincubated membranes were parallel and the apparent dissociation constants were not significantly different. Thus Ro 03-7894 could be considered to form an apparent irreversible or slowly dissociable complex with the β-adrenoceptor binding sites that could not be reversed by a washing procedure that had completely reversed propranolol. Admittedly further washing could have been attempted, but the two washings were adequate for complete removal of propranolol and others have found 10^{-7} M propranolol to be reversed by 2 washings [5].

Some variability of the B_{max} and K_D values was found between individual experiments in a group.

However, the differences between control and antagonist-treated membranes were readily demonstrated by statistical analysis of the mean values by both Student's *t*-test and the non-parametric Mann–Whitney U-test. Furthermore, it should be pointed out that antagonist pretreatments were always compared with controls performed simultaneously on the same membrane fraction under identical conditions. The results are therefore independent of any variations between control membranes. It was therefore appropriate to compare Ro 03-7894 with propranolol by calculating the individual differences between test and the corresponding control K_D and B_{max} values. The mean values showed that the reduction in binding sites in the presence of Ro 03-7894 or after its washout was significantly greater than the changes with propranolol.

β-Adrenoceptor antagonists have previously been described which are also thought to antagonize in an apparently irreversible manner. The activities of both FM 24 [8–11] and NHNP-NBE [12, 13] have been examined using radioligand binding techniques. When rat heart membranes were examined in the presence of FM 24 the total number of binding sites was reduced [5]. However, when incubated with particular fractions of cultured glioma C6 cells, the Scatchard analysis of [^{3}H]DHA binding in its presence was shifted in a manner characteristic of a competitive inhibition [6]. This contrasts with Ro 03-7894 in the presence of which there was a reduced B_{max} and apparently increased affinity. No explanation is available for this apparent increase in affinity, but when free Ro 03-7894 was removed by washing, the K_D values were identical to those in control untreated membranes. Thus the presence of the free Ro 03-7894 might account for the change in K_D value.

NHNP-NBE has been shown to behave in a similar fashion to FM 24 and Ro 03-7894 in that it reduced the number of binding sites in cat cardiac membranes and this was resistant to washout 5 times [10].

The antagonism of isoprenaline-induced positive inotropic responses of guinea-pig isolated papillary muscle by Ro 03-7894 was examined at the same temperature. After a 30 min incubation with Ro 03-7894 followed by thorough washout of the organ bath, the dose–response curve to isoprenaline was depressed. This failure to attain the same maximum response is indicative of apparent irreversible antagonism [27] and confirms previous observations with Ro 03-7894 using guinea-pig isolated left and right atria [11, 12]. These earlier studies have shown that this is not due to a non-specific depression of cardiac contractility since responses to histamine and calcium are not antagonized [11].

Comparisons between the antagonism of radioligand binding and of biological responses have also been made with FM 24 and NHNP-NBE. FM 24 was shown to irreversibly antagonize isoprenaline-stimulated adenylate cyclase activity of rat heart [5], liver [7] and C6 glioma cell membranes [6] after repeated washing. The activity of NHNP-NBE, like Ro 03-7894, was examined on isolated papillary muscles, but from the cat [10]. However, the results appear to differ in one important respect; increasing concentrations of NHNP-NBE produced progressive parallel displacement of the dose–response curves without depression of the maximum response. It is conceivable that the 30 min washout time quoted was not sufficient to remove free antagonist from the bath and that there was some additional competitive antagonism. Such a mechanism has been proposed for short washout times (20 min) of Ro 03-7894 [11].

In conclusion, this study has demonstrated that affinity values of propranolol for cardiac ventricular adrenoceptors are comparable when determined pharmacologically in papillary muscles and by radioligand binding. Furthermore, both pharmacological analysis and β-adrenoceptor binding techniques have shown that Ro 03-7894 behaves as an apparently irreversible or slowly dissociable antagonist when compared with propranolol. This activity has features in common with that of the antagonists FM 24 and NHNP-NBE, although certain properties could be identified that distinguish between them.

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