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THE β ADRENERGIC RECEPTOR OF RAT CORPUS LUTEUM MEMBRANES

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Abstract—A comparison has been made between the β receptors of rabbit heart membranes and superovulated rat corpus luteum in terms of adenylate cyclase activation and [^{3}H]dihydroalprenolol binding. Agonist activation and binding agree in rank order, which is isoprenaline > noradrenaline $\geqslant$ adrenaline > salbutamol for heart, and isoprenaline > adrenaline > salbutamol > noradrenaline for corpus luteum. The β_1 selective blockers, practolol, atenolol and ICI 89,406, are all less potent in the corpus luteum than in the heart but the order of potency is not the same in corpus luteum as was previously found in the uterus, and salbutamol, which is a full agonist in uterus, is only a partial agonist in corpus luteum. These findings do not agree with a clear cut separation of receptors into classical β_1 and β_2 types.

Since the original subdivision of β-adrenoceptors into β_1 and β_2 types using various agonists [1, 2], support for this classification has accumulated from pharmacological investigations with the β_1-selective blockers practolol [3], atenolol [4], metoprolol [5] and the β_2-selective blocker H35/25 [6]. Biochemical studies using broken cell preparations have largely agreed with the receptor groupings obtained using whole organ responses [7]. Burges and Blackburn [8] used adenylate cyclase as a marker of β-receptor activity and with a number of selective agonists and blockers confirmed rat heart to be β_1 and lung to be β_2 type tissues. Mayer [9] using a similar technique proposed that dog heart contained β_1 receptors and liver β_2 receptors.

In a previous communication [10] we pointed out that most of these authors wrongly used antagonist ED_{50} values to compare receptor types. In that work we used the adenylate cyclase of purified plasma membranes from rabbit heart, lung and uterus as an index of β-receptor subtype, and with the aid of Schild plots [11] determined accurate dissociation constants for a series of β-blockers. We proposed that the majority of cells in heart and lung give a β_1-like response whereas uterus shows β_2 characteristics. We have now extended this work to study β-adrenoceptors in the rat corpus luteum membranes described by Birnbaumer *et al.* [12], using both measurements of adenylate cyclase activity and direct binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) [13]. In this communication we describe the characterization of the β-adrenoceptor in rat corpus luteum in terms of its affinity for various β_1 and β_2-selective drugs and show that its specificity for these compounds is characteristic of a β_2 system. Corpus luteum is a much more tractable system than uterus, showing higher stimulation of adenylate cyclase with isoprenaline and excellent reproducibility in both adenylate cyclase and direct binding studies. In an accompanying paper [14] we present data based on heart and corpus luteum membranes which substantiate our earlier ideas that biochemically distinct β-adrenoceptor subtypes do exist.

MATERIALS AND METHODS

Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from the Sigma Chemical Company, Kingston-upon-Thames, Surrey. [^{3}H]dihydroalprenolol ([^{3}H]DHA, 32.6 or 46 Ci/m-mole) was obtained from New England Nuclear, Boston, MA, U.S.A. All other materials came from either commercial sources or as stated in [10]. Propranolol (Inderal), practolol (Eraldin) and atenolol (Tenormin) are made by I.C.I. Limited.

Preparation of rat corpus luteum membranes. The preparation of rat corpus luteum membranes was modified from the method of Birnbaumer *et al.* [12]. Immature 28-day old female rats (Alderley Park strain) were injected subcutaneously with 50 i.u. PMSG, then 56 hr later with 50 i.u. hCG. Five days after dosing with hCG, ovaries from groups of 20 superovulated rats were excised and placed in ice-cold 10 mM Tris/HCl, 1 mM ethyleneglycol-bis-(β-amino ethyl ether) *N,N'*-tetraacetic acid (EGTA), 0.5 mM $MgSO_4$, 27% (w/v) sucrose buffer, pH 7.4 (27% sucrose buffer). All preparative procedures thereafter were done at 0–4°. The ovaries (5 g), containing about 90 per cent corpus luteum tissue [12], were homogenised in 5 vol. 27% sucrose buffer with a Polytron PT 10 homogeniser set at mark 4 for two periods of 20 sec. The homogenate was then diluted 4-fold with 27% sucrose buffer and centrifuged for 5 min at 160 *g* using an 8 × 50 ml SS34 rotor in a Sorvall RC5 centrifuge to sediment large fragments of cell debris. The supernatant was filtered through 1 layer cheesecloth (0.5 mm mesh) and recentrifuged at 10,000 *g* for 45 min in the SS34 rotor. The resulting pellets, containing a crude membrane fraction, were resuspended in 60 ml 27% sucrose buffer using a Teflon–glass homogeniser (6–8 strokes). This preparation was used without further purification for both adenylate cyclase and direct binding studies.

Preparation of rabbit heart and uterus membranes. The preparation of rabbit heart and uterus plasma membranes is described elsewhere [10].

Direct binding studies using [^{3}H]*DHA*. The direct binding technique used to measure the affinities of adrenergic drugs for β-adrenoceptors was similar to that described by Lefkowitz [15]. Heart and uterus membranes were suspended at 0.1–0.5 mg protein/ml in the buffer (5 mM Tris/HCl, pH 7.4, sp.gr. 1.136) used for density gradient centrifugation [10], while corpus luteum membranes were used at 1–3 mg/ml protein in the 27% sucrose buffer. [^{3}H]DHA and the various drugs studied were dissolved in 5 mM Tris/HCl, 1 mM EGTA, 0.5 mM $MgSO_4$, 0.25 M sucrose, pH 7.4 ("5 mM Tris/0.25 M sucrose buffer"). Each incubation containing 800 μl membranes, 100 μl [^{3}H]DHA (12–15,000 c.p.m., 0.5–0.7 nM final concentration) and 100 μl drug was incubated at 30° for 20 min after which receptor-bound and free drug were separated by filtration, using Whatman GF/F 2.4 cm filters for heart and uterus membranes and GF/C filters for the more crude corpus luteum preparations. The filters were washed with three 6 ml aliquots of 5 mM Tris/0.25 M sucrose buffer at room temperature, placed in scintillation vials containing 3.5 ml PCS scintillator (Amersham-Searle), and counted in a Philips Automatic Liquid Scintillation Analyser after being allowed to equilibrate for several hours.

Non-specific [^{3}H]DHA binding was defined as the radioactivity measured in the presence of 10^{-5} M unlabelled alprenolol (without subtracting the filter blank) and at 0.5 nM labelled DHA this was about 30–50 per cent of the total binding. In some cases 10^{-7} M unlabelled alprenolol or 200 μM isoprenaline were used for comparison and there was little difference from 10^{-5} M. Subtraction of this figure from the total radioactivity bound in presence of [^{3}H]DHA alone gave the maximum specific binding and all points on each dose–response curve were expressed as a percentage of this maximum and plotted on log probit paper to determine the ED_{50}. A dose–response curve to alprenolol was constructed in each experiment, and these accumulated alprenolol data were used to obtain a mean 50 per cent displacement value which was taken as equivalent to the alprenolol K_D to avoid the complexities of analysing curved Scatchard plots (see below). Dissociation constants for the test drugs (drug K_D) were then determined using the relationship [16]:

$$\text{Drug } K_D = \frac{\text{Drug } ED_{50}}{\text{Alprenolol } ED_{50}} \times \text{Alprenolol } K_D$$
for same experiment

Adenylate cyclase incubations and estimation of cyclic AMP. These techniques are described elsewhere [10]. 25 μg Corpus luteum protein was used for each adenylate cyclase incubation. K_{act} is used to define the concentration of an agonist which produces 50 per cent of its maximal response and K_I is the dissociation constant for an antagonist as obtained from Schild plots. K_D is used for the dissociation constant obtained as above from binding studies (cf. Maguire, Ross and Gilman [17]).

RESULTS

In this investigation, binding of [^{3}H]DHA to heart or corpus luteum membranes has been shown to be rapid and easily reversible by a large excess of unlabelled

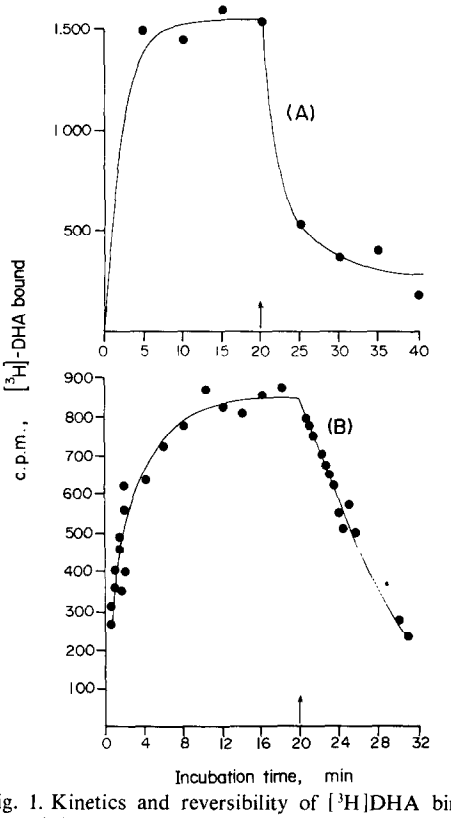

Fig. 1. Kinetics and reversibility of [^{3}H]DHA binding to heart (A) and corpus luteum (B) membranes: 7 x 10^{-10} M [^{3}H]DHA added at time zero; 10^{-5} M cold alprenolol added at ↑.

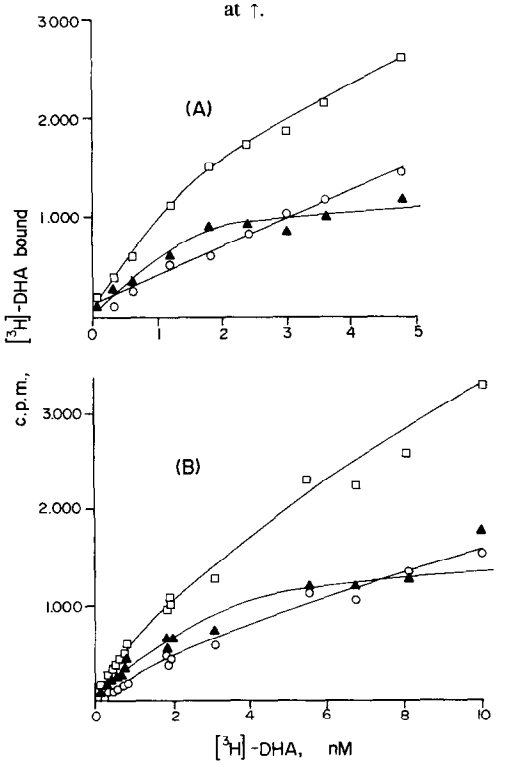

Fig. 2. Saturation of [^{3}H]DHA binding to heart (A) and corpus luteum (B) membranes. □ Total binding measured in the absence of unlabelled alprenolol; ○ non-specific binding measured in the presence of 10^{-5} M unlabelled alprenolol; ▲ specific binding (difference between total and non-specific).

Table 1. Comparison of agonist potencies in heart and corpus luteum membranes determined by adenylate cyclase and direct binding methods

	Adenylate Cyclase				Binding			
	Heart*		Corpus luteum		Heart		Corpus luteum	
	K_{act} (M)	Potency ratio	K_{act} (M)	Potency ratio	K_D (M)	Potency ratio	K_D (M)	Potency ratio
(±)-Isoprenaline	1.55×10^{-7}	1.0	3.6×10^{-7}	1.0	1.20×10^{-7}	1.0	2.49×10^{-8}	1.0
(−)-Noradrenaline	1.23×10^{-6}	7.9	2.6×10^{-5}	72.0	7.62×10^{-7}	6.4	2.20×10^{-6}	88.4
(−)-Adrenaline	1.90×10^{-6}	12.3	1.7×10^{-6}	4.7	1.09×10^{-6}	9.1	1.90×10^{-7}	7.6
Salbutamol	1.54×10^{-4}	994.0	3.6×10^{-6}	10.0	1.70×10^{-6}	14.2	2.52×10^{-7}	10.1

* Coleman and Somerville [10].

alprenolol (Fig. 1). Saturation of the specific binding sites in the heart is sharply defined, with half maximal occupation at about 1 nM, though in corpus luteum the saturation takes place over a wider range of ligand concentrations (Fig. 2), and Scatchard analysis suggests the presence of multiple high affinity binding sites with apparent constants ranging from 1 to 5 nM, or else the existence of negative cooperativity. In this situation the true constants and maximum binding for each site are no longer given simply by the slopes and intercepts of the Scatchard plots, which are composites of the values for all sites (Klotz and Hunston [18]). The mean ED_{50} obtained by alprenolol displacement in corpus luteum (3.7 nM, Table 2) is in the same range as these apparent values measured by association, but the ED_{50} value for heart (6.1 nM) is somewhat higher than the 1 nM found in association experiments.

Incubation of membranes with [^{3}H]DHA and increasing concentrations of L- and D-propranolol (Fig. 3) shows stereospecific displacement of the radioligand with preference for the L-isomer.

Apart from salbutamol the agonist potencies for heart membranes show good agreement using either the adenylate cyclase or direct binding techniques, both in magnitude and order: D-L-isoprenaline > L-noradrenaline ≥ L-adrenaline (Figs. 4 and 5 and Table 1). The order of agonist potencies using the corpus luteum preparation: D-L-isoprenaline >L-adrenaline > salbutamol> L-noradrenaline is identical whether measured by adenylate cyclase or binding, but in this tissue all agonists are 10 times more potent in the binding system than in adenylate cyclase activation. Salbutamol is a partial agonist in heart [10] and corpus luteum, giving 60 per cent and 42 per cent respectively of the maximum adenylate cyclase response to isoprenaline. Using heart membranes, salbutamol is one hundred times

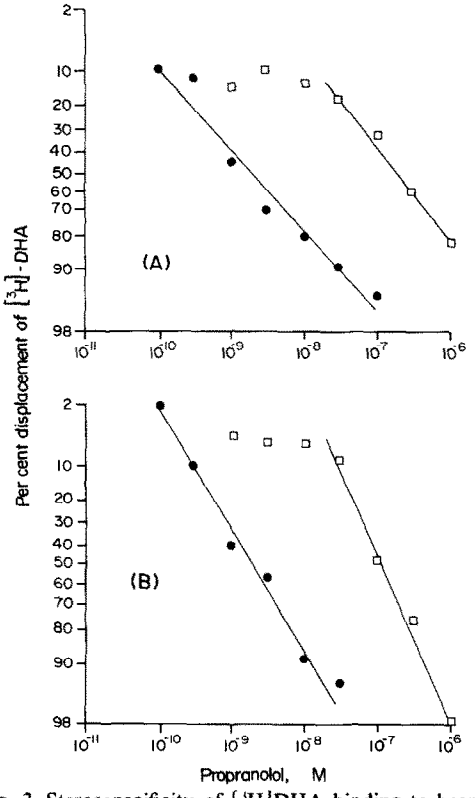

Fig. 3. Stereospecificity of [^{3}H]DHA binding to heart (A) and corpus luteum (B) membranes: log-probit scale—●, L-propranolol; □, D-propranolol.

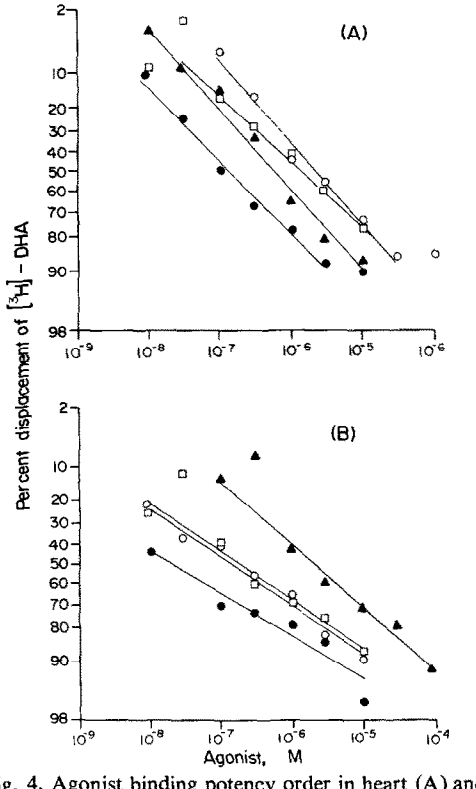

Fig. 4. Agonist binding potency order in heart (A) and corpus luteum (B) membranes: displacement of [^{3}H]DHA by DL-isoprenaline ●, L-noradrenaline ▲, L-adrenaline □, and salbutamol ○.

Table 2. Comparison of β-blocker dissociation constants determined using adenylate cyclase, direct binding and isolated tissues (Nos of determinations in brackets)

	Adenylate cyclase (K_I)			Binding (K_D)			Organ bath (K_I)*	
	Heart	Uterus	Corpus luteum	Heart	Uterus	Corpus luteum	Atrium‡	Trachea§
Alprenolol	1.25×10^{-8} (1)	—	2.8×10^{-9} (1)	$6.1 \pm 0.55 \times 10^{-9}$ (20)	$5.80 \pm 0.53 \times 10^{-9}$ (4)	$3.7 \pm 0.51 \times 10^{-9}$ (17)	4.3×10^{-9} (9)	—
Propranolol	$3.4 \pm 0.5 \times 10^{-9}$†	$1.5 \pm 0.6 \times 10^{-9}$†	1.35×10^{-9} (2)	4.2×10^{-9} (2)	—	3.56×10^{-9} (2)	5.0×10^{-9} (6)	1.7×10^{-8} (8)
Practolol	$3.2 \pm 0.4 \times 10^{-6}$†	5.4×10^{-5}†	6.2×10^{-5} (2)	5.36×10^{-7} (2)	2.74×10^{-5} (2)	7.16×10^{-5} (2)	3.9×10^{-7} (8)	2.3×10^{-5} (5)
Atenolol	1.3×10^{-6}†	$6.7 \pm 2.0 \times 10^{-5}$†	$1.27 \pm 0.10 \times 10^{-5}$ (5)	3.69×10^{-7} (2)	1.04×10^{-5} (2)	1.48×10^{-5} (2)	5.4×10^{-8} (5)	5.9×10^{-6} (5)
ICI 89,406	$7.5 \pm 4.7 \times 10^{-9}$ (6)	$5.7 \pm 1.8 \times 10^{-8}$ (4)	$1.89 \pm 0.20 \times 10^{-7}$ (4)	3.73×10^{-9} (2)	—	4.02×10^{-7} (2)	3.6×10^{-9} (3)	7.3×10^{-7} (5)
H 35/25	6.1×10^{-6} (2)	—	4.5×10^{-7} (2)	1.82×10^{-6} (2)	—	4.0×10^{-7} (2)	—	—

* [10], updated by personal communication from Dr. J. Wale.
† From [10] determined using Schild Plots (Practolol in uterus has a Schild Plot slope significantly < 1).
‡ Spontaneous beating rate of G.P. right atria.
§ Relaxation of G.P. tracheal smooth muscle.

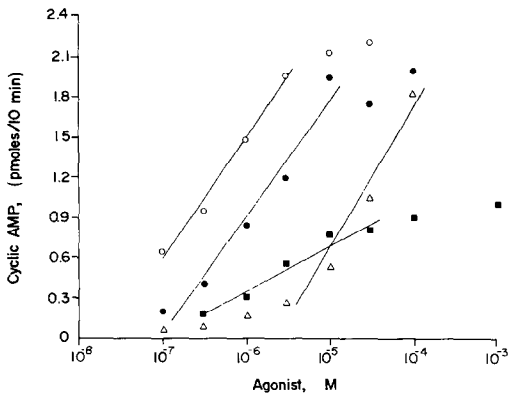

Fig. 5. Increases in cyclic AMP produced in response to (DL)-isoprenaline (○); (L)-adrenaline (●); (L)-noradrenaline (△) and salbutamol (■). Incubation medium (200 μl 40 mM Tris–HCl pH 7.4, $MgSO_4$ 6 mM, EGTA 0.1 mM, theophylline 9 mM, ICI 63,197 1.5 mM, Tris ATP 1.8 mM, creatine phosphate 20 mM, 200 μg/ml creatine kinase) was added to 20 μl agonist at the appropriate concentrations and preheated to 30°. The reaction was started by adding 20–60 μg corpus luteum protein in a 20 μl aliquot and incubated at 30° for 10 min. 100 μl of 160 mM HCl was added to stop the reaction and after heating to 95° for 6 min, Tris buffer (100 μl of 200 mM) was added to adjust to pH 7.4. After a further heating at 95° for 7 min and centrifugation, an aliquot of supernatant was taken for the cAMP assay.

more potent in [^{3}H]DHA displacement than in adenylate cyclase activation (Table 1).

There is close agreement in the corpus luteum between β-agonist dissociation constants measured using either the adenylate cyclase or the [^{3}H]DHA displacement technique (Table 2, Figs. 6 and 7), and the adenylate cyclase dose–response curves obtained from corpus luteum membranes illustrate the excellent parallel displacement, low scatter and routine 10-fold stimulation by isoprenaline achieved in this system. Agreement between K_D and K_i is not so good when heart membranes are used, however, affinities being generally greater for binding than for cyclase activation. The further implications of this are discussed in an accompanying paper [14]. The pharmacologically β_1-selective blockers, practolol, atenolol and ICI 89,406*, are more potent in heart than corpus luteum implying that corpus luteum β-receptors are similar to those of uterus and should be classified as a β_2 subtype (Table 3). This interpretation is complicated by the Schild plots for ICI 89,406 and atenolol illustrated in Fig. 8 (a and b) where, although their greatest potency is with heart membranes, atenolol is more potent in corpus luteum than uterus ($P < 0.005$) whereas ICI 89,406 is more potent in uterus than corpus luteum ($P < 0.0025$). These findings cannot be substantiated by binding data since the uterus preparation gave very scattered results for ICI 89,406 displacement of [^{3}H]DHA.

DISCUSSION

In an earlier paper [10] we found that the responses of rabbit lung and heart cyclase to β_1 selective antagonists were similar, contrary to what might have been expected from pharmacological evidence. It now appears that the rabbit was an unfortunate choice for this comparison since subsequent work by Dr. S. R. Nahorski (personal communication) on the analysis of binding curves has shown that whereas rat, guinea pig and bovine lung contain mainly β_2 receptors (less than 30 per cent β_1), the rabbit has a high proportion (~60 per cent) of β_1. Species differences are thus very important in determining β receptor character.

The present communication describes the preparation of super-ovulated rat corpus luteum membranes and the classification of their β receptor type using both adenylate cyclase and direct binding techniques.

Binding of [^{3}H]DHA to heart and corpus luteum membranes satisfies all the requirements for specific binding to the β receptor. Binding was rapid and easily reversible, saturable and stereospecific, being displaced preferentially by the L-isomer of propranolol. Using the adenylate cyclase system, extremely reproducible quantitative K_i measurements were possible with the corpus luteum membrane β receptor.

The agonist potency order: DL-isoprenaline > L-noradrenaline ≥ L-adrenaline > salbutamol determined for the heart membranes using both adenylate cyclase and direct binding methods closely resembles the classical pharmacological β_1 sub-group profile, whereas the corpus luteum displays the β_2 receptor order DL-isoprenaline > L-adrenaline > salbutamol > L-noradrenaline.

When the corpus luteum preparation is used, the K_{act} (cyclase) is 10-fold larger than the K_D (binding), although the difference is not so great for heart membranes. It has been shown that agonist binding affinity is decreased by GTP (see, for example, Maguire, Ross and Gilman [17]), and while the binding studies reported here were conducted in the absence of GTP, we have found in a few experiments that GTP markedly reduces the affinity of agonists for corpus luteum, but that it has much less effect on heart. This would account for the K_{act}/K_D differences reported above (since we have also found that GTP does not affect cyclase activation in these preparations), and it suggests that GTP affects coupling between receptor binding and end-effect more strongly in the corpus luteum system than in the heart.

The pharmacologically non-specific β blocker propranolol remains non-selective when measured on heart and corpus luteum membranes. The β_1 selective blockers, practolol, atenolol and ICI 89,406, are all less potent in the corpus luteum, implying that corpus luteum contains β_2 receptors. This point is reinforced by the β_2 selective blocker H35/25 being more potent in the corpus luteum than the heart membranes, and it substantiates our previous conclusion that drugs which act selectively in whole tissues retain this selectivity when the exposed membrane bound receptors are directly available for drug binding.

The apparently clear-cut β receptor subdivision outlined above becomes inadequate when the corpus luteum β_2 receptor is compared with the uterus receptor, which was also determined as a β_2 type [10]. Although in most aspects they both appear distinct from the heart in subtype, a highly significant difference in β blocker potency order is observed between these β_2 systems. Moreover, salbutamol, a classical β_2 full agonist, is a

* 1-(2-cyanophenoxy)-3-(β-phenyl-ureido-ethyl-amino)-2-propanol.

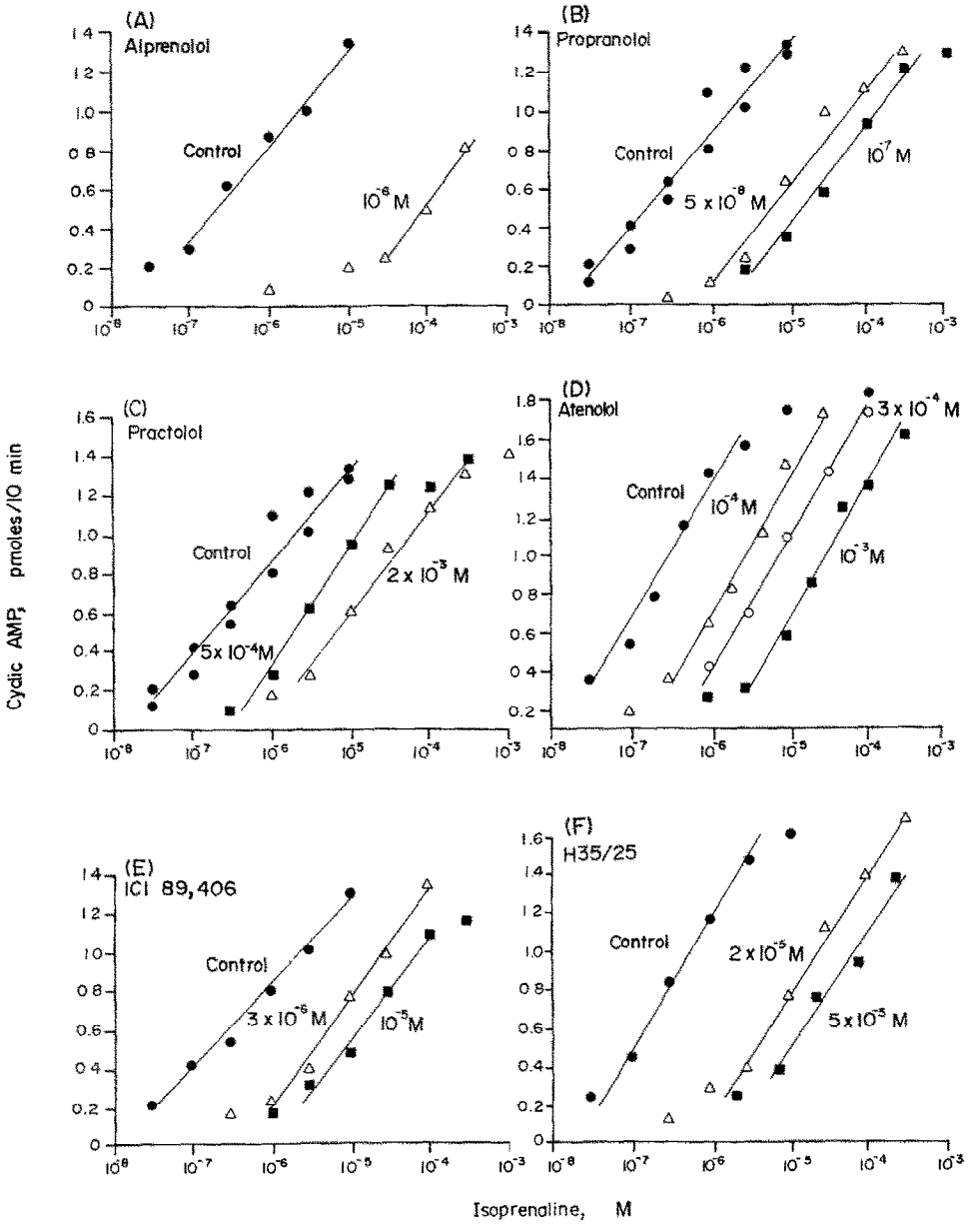

Fig. 6. (DL)-Isoprenaline dose–response curves for adenylate cyclase: (DL)-isoprenaline alone (control) and in the presence of varying amounts of alprenolol (A), propranolol (B), practolol (C), atenolol (D), ICI 89,406 (E) and H35/25 (F) using corpus luteum membranes. The incubation conditions are as described in Fig. 5. 20 μl of antagonist being added to 20 μl agonist before addition of the incubation medium.

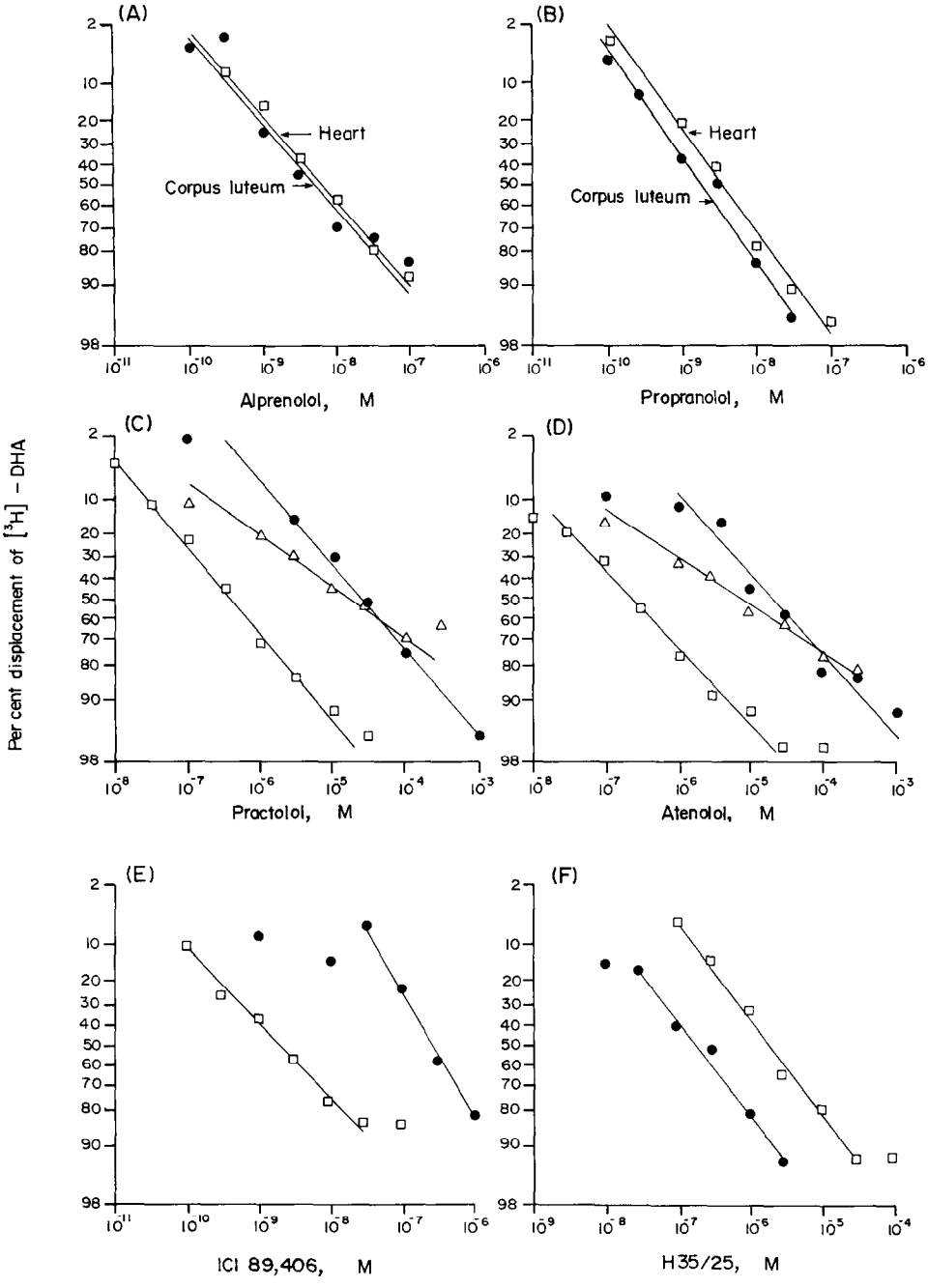

Fig. 7. Displacement of [^{3}H]DHA from heart (□), uterus (△) and corpus luteum (●) membranes by alprenolol (A), propranolol (B), practolol (C), atenolol (D), ICI 89,406 (E) and H35/25 (F). For conditions see text.

Table 3. Selectivity ratios determined from dissociation constants using membrane preparations and isolated tissues

	Heart/corpus luteum		Heart/uterus		Uterus/corpus luteum		Atrium*/trachea†
	Cyclase	Binding	Cyclase	Binding	Cyclase	Binding	Organ bath
Alprenolol	0.22	0.61	—	0.95	—	0.64	—
Propranolol	0.4	0.86	0.4	—	0.9	—	3.4
	n.s.		n.s.		n.s.		
Practolol	19.4	133.58	16.9	51.12	1.1	2.61	59.0
	$P < 0.0005$						
Atenolol	9.8	40.11	51.5	28.18	0.19	1.42	109.0
	$P < 0.0005$		$P < 0.001$		$P < 0.05$		
ICI 89,406	25.2	107.78	7.6	—	3.32	—	203.0
	$P < 0.0005$		$P < 0.0025$		$P < 0.0025$		
H 35/25	0.07	0.22	—	—	—	—	—

Significance values between dissociation constants calculated from individual dose–response curves using Student's *t* test.

* Spontaneous beating rate of g.pig right atria.

† Relaxation of g.pig tracheal smooth muscle.

partial agonist in the corpus luteum but a full agonist in the uterus as mentioned above, although its potency relative to other agonists on corpus luteum membranes fits the normal β_2 order. These results cannot be totally explained by suggesting that each tissue contains a different mixture of two distinct β receptor types (cf. Carlsson *et al.* [19]; Ariens and Simonis [20]) but instead strongly imply that the β_2 receptor type is not a unique drug recognition site.

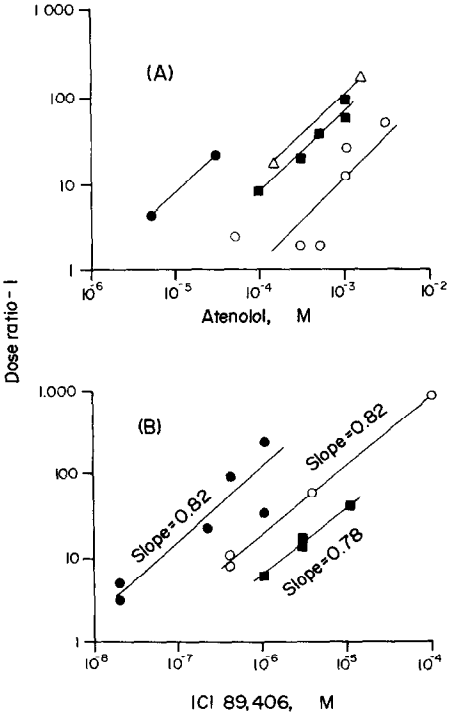

Fig. 8. A. Schild plots for atenolol, using heart (●), trachea (△), uterus (○) and corpus luteum (■) membranes and isoprenaline as agonist. Data for heart, trachea and uterus are from [10]. Corpus luteum cyclase incubations as described in Fig. 6. The lines are the best fit of slope unity. B. Schild plots for ICI 89,406 using heart (●), uterus (○) and corpus luteum (■) membranes and isoprenaline as agonist. The lines were drawn by least squares analysis.

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