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SPECIFIC BINDING OF [^{3}H]PRAZOSIN TO MYOCARDIAL CELLS ISOLATED FROM ADULT RATS

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Abstract—The characteristics of α-adrenoceptors in rat myocardium were investigated by specific binding of [^{3}H]prazosin to cells isolated from adult rat heart by perfusion with collagenase and hyaluronidase. The cells were incubated in Krebs–Ringer bicarbonate buffer gassed with 95% O_2 and 5% CO_2 at 31° with the appropriate concentrations of the different ligands. Non-specific binding was defined by the addition of 10^{-5} mole/l. phentolamine. The binding of [^{3}H]prazosin was saturable and reached equilibrium within 15 min. Scatchard analysis showed a straight line giving an apparent dissociation constant, K_d, equal to 155.9 ± 8.0 pmole/l. and a maximal number of binding sites equal to 76.7 ± 11.1 fmole/mg protein. Inhibition of specific [^{3}H]prazosin binding by different adrenergic blockers showed the order of potency characteristic of α_1-adrenoceptors: prazosin ≫ phentolamine > yohimbine ≫ propranolol. Inhibition by adrenergic agonists showed the order of potency: adrenaline > noradrenaline = phenylephrine > isoprenaline. The same orders of potency were observed in the presence of propranolol. However, propranolol slightly decreased the affinity for noradrenaline and phenylephrine. Hofstee analyses of the inhibition curves showed two binding components for all ordinary α-adrenoceptor blockers and agonists including unlabelled prazosin. In contrast, [^{3}H]prazosin showed only one binding component. Both binding components were of the α_1-adrenoceptor subtype according to the order of potency of blockers. The different ligands had different affinity ratios for the two binding components giving them different profiles. Trifluoperazine, a phenothiazine compound, also had high affinity for the [^{3}H]prazosin binding sites. This drug, however, apparently detected one class of binding sites only, as interpreted from the Hofstee analysis. Hill analyses of the inhibition data consistently yielded Hill constants, n_H, in the range 0.75–0.85 except for [^{3}H]prazosin, where $n_H = 1.02$ and for trifluoperazine, where $n_H = 1.07$. Although the two binding components may serve different functions, it seems impossible at present to relate the negative and the positive inotropic components, respectively, of the α-adrenergic inotropic response observed in functional studies only to one or the other binding component.

Specific binding of radioligands has been successfully used to characterize a variety of receptors, especially the adrenergic receptors for which there exists a great number of labelled and unlabelled ligands with different binding profiles [1]. The radioligand initially used for studying α-adrenoceptors was [^{3}H] dihydroergocryptine (DHE)* [2]. Later, several agonists and blockers became available as radioligands. By employing different agents, it is now well accepted that α-adrenoceptors can be divided into two different subgroups: α_1 and α_2 ([3]; for a review see ref. [4]). Originally, the concept was that α_2-adrenoceptors probably were situated presynaptically (prejunctionally) regulating neurotransmitter release while α_1-adrenoceptors were situated on the effector cell (post-junctionally) mediating the physiological response [3, 5, 6]. Later this concept was modified by evidence which also postulates several types of post-synaptic α-adrenoceptors [7–14]. In membranes from the heart, Guicheney *et al.* [15, 16] found two types of α-adrenoceptors, classified as α_1-and α_2-receptors, respectively, using [^{3}H] DHE as the radioligand. U'Prichard *et al.* [17] found that inhibition of [^{3}H]DHE binding by prazosin displayed a shallow biphasic curve. Other groups [18, 19], also using [^{3}H]DHE, found one type of binding sites. Karliner *et al.* [20] found one type of α-adrenoceptors in guinea-pig heart classified as α_1, using [^{3}H]prazosin as the radioligand. Williams *et al.* [21] also found predominantly the α_1-receptor subtype. All these studies were performed with membrane preparations.

In functional studies in rat papillary muscles, we found differential effects of prazosin and phentolamine on the α-adrenergic response to stimulation by phenylephrine in the presence of propranolol [22]. Under conditions where the positive inotropic component was blocked by about the same degree, the transient negative inotropic component was more susceptible to blockade by phentolamine than by prazosin. This might indicate that the adrenoceptors mediating these two effects were different and accordingly might be recognized as different entities. We therefore found it of interest to examine binding characteristics of α-adrenoceptor ligands. Mostly, membrane preparations and artificial buffer systems are used in binding studies. In order to characterize the α-adrenoceptors in rat heart in a more integrated system, myocardial cells isolated from adult rat heart and suspended in Krebs–Ringer bicarbonate buffer were used. [^{3}H]Prazosin was used as labelled ligand because of its apparent low non-specific binding. Preliminary results have been presented elsewhere [23].

* Abbreviations: DHE, dihydroergocryptine; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*, *N'*-tetraacetic acid.

MATERIALS AND METHODS

Isolation of cells. The cells were isolated from adult rat heart by the method described by Moustafa *et al.* [24] modified according to Farmer *et al.* [25] and Clark *et al.* [26]. Male Wistar albino rats weighing 230–300 g were used. During ether anaesthesia, the hearts were isolated and perfused through the aorta with a nominally calcium-free salt solution buffered with bicarbonate and equilibrated with 95% O_2 and 5% CO_2. The salt solution contained (in mmole/l.): NaCl 122.0, KCl 3.0, KH_2PO_4 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, glucose 10 and 0.05% bovine serum albumin. After 10 min wash-out and equilibration, the hearts were perfused for 25 min with 30 ml of the same solution containing 100 IU/ml collagenase and 80 IU/ml hyaluronidase. The coronary flow was adjusted to 6–7 ml/min during the enzyme treatment. After enzyme treatment, the same salt solution precooled to 0–4° and supplemented with 0.5 mmole/l. EGTA was run through the hearts without recirculation for 5 min in order to inhibit and to wash out the enzymes. After the wash-out period, the ventricles were cut down from the perfusion apparatus and the cells dispersed in 2–3 ml of the wash-out solution by mincing the tissue and gently stirring for 5 min. The cell suspension was also gassed during the stirring to avoid alkalosis in the medium. The resultant mixture of cells and tissue was filtered through cheesecloth and was used without further purification. The cell suspension was diluted to the appropriate volume and stored at 0–4° until use, not later than 15 min after filtration.

Incubation of cells and binding assay. The cells (corresponding to 1–2 mg protein) were incubated in a total volume of 1 ml in 25 ml siliconized Erlenmeyer flasks in a Grant shaking water bath set at 100 strokes/min and 31°. The cells were incubated in the salt solution given above containing 0.5 mmole/l. EGTA and equilibrated with 95% O_2 and 5% CO_2 (pH 7.4) and with the appropriate concentrations of the different ligands. During incubations with agonists, 10^{-4} mole/l. ascorbate was present. This concentration of ascorbate did not interfere with the binding of the labelled ligand. Incubations were usually carried out for 15 min except in the time-course experiments where the incubations were stopped at appropriate times. Separation of free from bound ligand was achieved by adding 4 ml of the incubation medium (gassed) to the flasks (22°). The resulting suspension was immediately filtered through Whatman GF/A glass fibre filters (vacuum 17 kPa). The filters were then washed with 4 ml + 12 ml of the same solution (22°). The whole separation procedure was completed within 15–20 sec. The filters were placed in counting vials and dried at 40° for 2 hr. Then the radioactivity retained on the filters was eluted by the addition of 0.1 ml of 0.5 mole/l. HCl. Scintillation fluid (6 ml; Hydroluma ®) was added and the vials were shaken for 30 min. The radioactivity was then determined in a Packard liquid scintillation spectrometer, model 2450.

Specific binding was defined as the difference in bound radioactivity in the absence compared to the presence of 10^{-5} mole/l. phentolamine. This concentration of phentolamine, which displaces more than 99% of the radiolabelled prazosin, is about 550 times its observed average equilibrium dissociation constant (K_d). Non-specific binding was less than 10% of total binding, except in saturation experiments where the non-specific binding amounted to 15–20% at the highest concentrations used.

Protein determination. Cell protein was determined according to Lowry *et al.* [27] using bovine serum albumin as standard.

Calculations. Saturation experiments with increasing concentrations of radioligand were analysed according to Scatchard [28]. The apparent affinity of the labelled ligand was calculated from the slope of the curve and the maximal number of binding sites, expressed as fmole/mg cell protein, was calculated from the intercept with the abscissa.

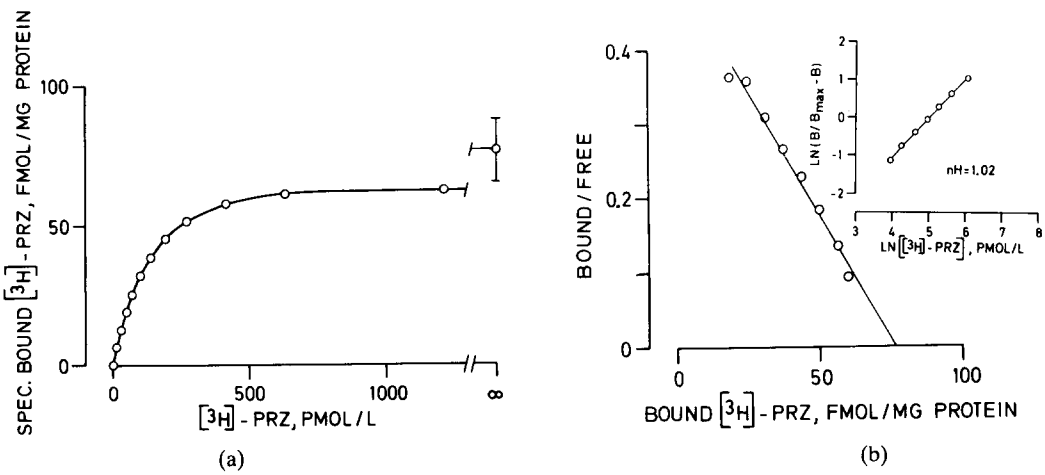

Fig. 1. (a) Specific binding of [^{3}H]prazosin to isolated myocardial cells as a function of increasing concentrations of the radioligand. The incubations were carried out for 15 min at 31°. Bound radioligand (ordinate) is expressed as fmole/mg protein and free concentration (abscissa) in the incubate as pmole/l. The amount of bound radioligand at infinite concentration (∞) is taken from the Scatchard analysis. S.E.M. is indicated at the calculated maximal value; n = 3. (b) Scatchard analysis of the data given in (a). Ordinate: bound/free radioligand. Abscissa: bound radioligand/mg protein. The inset shows the Hill analysis of the data giving a Hill coefficient (n_H) = 1.02.

The association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) for [^{3}H]prazosin were calculated from the time association curve by using the equation: $dB/dt = k_{+1} \times (S-B) \times (B_{max}-B) - k_{-1} \times B$, where S is the concentration of [^{3}H]prazosin, B is the amount of [^{3}H]prazosin bound at each time and B_{max} is the maximal amount of [^{3}H]prazosin that can be bound. The following approximations were made: (1) $dB/dt \approx \Delta B/\Delta t$, where $\Delta B/\Delta t$ is the average slope in each individual interval of the curve given in Fig. 2; (2) $S \gg B$. The equation then obtained is $\Delta B/\Delta t = -(k_{+1} \times S + k_{-1}) \times B + k_{+1} \times S \times B_{max}$, and can be solved graphically by plotting $\Delta B/\Delta t$ vs B. S can be used to calculate B_{max} from the data given in Fig. 1 and from the amount bound at equilibrium in Fig. 2. The association rate constant is then calculated from the intercept of the linear regression line with the $\Delta B/\Delta t$ axis and the dissociation rate constant then from the slope of this line. The advantage of this method is that both k_{+1} and k_{-1} can be determined under the same experimental conditions and at relatively low ligand concentrations, i.e. k_{-1} can be determined without the addition of a suprasaturating concentration of unlabelled ligand.

The inhibition curves were subjected to computer-assisted Hofstee analysis according to ref. [29] in order to detect possible subgroups of binding sites. IC_{50} values (the concentrations of inhibiting ligands reducing specific [^{3}H]prazosin binding by 50%) were obtained from the slopes of the components and the apparent affinities (K_d) calculated according to Cheng and Prusoff [30] using the equation $K_d = IC_{50}/(1 + S/K_m)$, where S is the concentration of [^{3}H]-prazosin and K_m is the K_d value for [^{3}H]prazosin. The apparent affinities of the different ligands studied were expressed as the negative logarithm of the dissociation constants (pK_d).

Both saturation and inhibition curves were subjected to Hill analysis [31] also in order to analyse the data with respect to subgroups of binding sites or interaction between binding sites.

The significance level of differences for the mean affinities were calculated according to the Wilcoxon two-sample test. A value of α equal to or less than 0.05 was considered to reflect significant differences.

Drugs and solutions. Prazosin hydrochloride was kindly supplied by Pfizer and phentolamine metanesulfonate by CIBA-Geigy Ltd. [^{3}H]Prazosin (33 Ci/mmole) was prepared at the Radiochemical Centre (Amersham, U.K.) and was a most generous gift from Dr. P. Hodges, Pfizer (Sandwich, U.K.). Yohimbine hydrochloride, 1-ascorbic acid and bovine serum albumin, fraction V, were obtained from Sigma Chemical Co. (St. Louis MO). Collagenase, type II (CLS II), and hyaluronidase were purchased from Worthington Biochemical Corp. (Freehold, NJ).

The other drugs were purchased through the Norwegian Medical Depot: (−)-noradrenaline bitartrate, (−)-adrenaline bitartrate, (−)-phenylephrine hydrochloride, (−)-propranolol hydrochloride, (±)-propranolol hydrochloride and (−)-isoprenaline hydrochloride. All other chemicals were of analytical grade.

Stock solutions were prepared in doubly distilled water and kept at −20°. Further dilutions in incubation buffer (containing 10^{-4} mole/l. ascorbate in the case of agonists) were prepared each experimental day. The dilutions were kept at 0–4° and in darkness until use. Repetitive experiments showed that drug solutions treated in this way were stable.

RESULTS

Binding of [^{3}H]*prazosin to isolated myocardial cells*

Specific binding of [^{3}H]prazosin showed a limited number of binding sites with a high apparent affinity

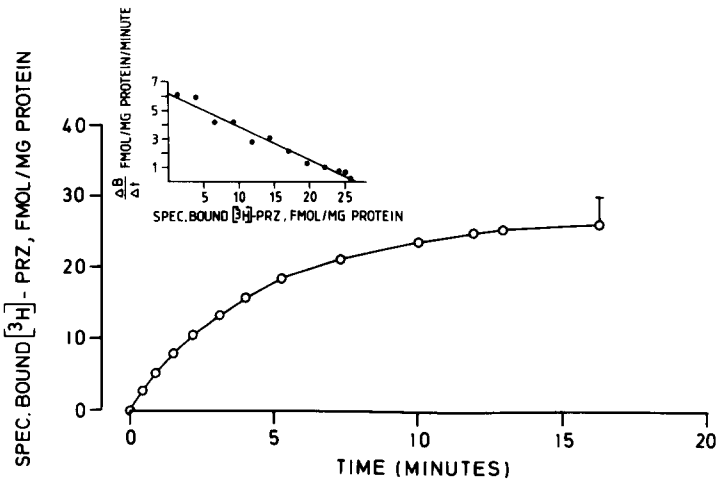

Fig. 2. Time course of the specific binding of [^{3}H]prazosin to isolated myocardial cells. Incubations were carried out for appropriate times at 31° and the reaction was stopped by filtration as described in Materials and Methods. [[^{3}H]Prazosin] = 150.5 pmole/l. Ordinate: amount bound expressed as fmole/mg protein; abscissa: time after the addition of labelled ligand. S.E.M. is indicated at the maximal value observed; $n = 4$. The inset shows the rate of increase of specifically bound [^{3}H]prazosin, $\Delta B/\Delta t$, expressed as fmole/mg protein/min (ordinate), plotted against specifically bound [^{3}H]prazosin (abscissa) in order to calculate the association and the dissociation rate constants (further explanation in the text).

for the ligand (Fig. 1a). Non-specific binding measured in the presence of 10^{-5} mole/l. phentolamine increased linearly in the concentration range tested (not shown). Scatchard analysis of the data for specific binding (Fig. 1b) showed a straight line indicating a single component of binding sites. The apparent dissociation constant (K_d) of three experiments was 155.9 ± 8.0 pmole/l. ($pK_d = 9.81$) and the calculated maximal number of binding sites from these experiments was 76.7 ± 11.1 fmole/mg protein.

Analysis of the data according to the Hill equation revealed a straight line with a Hill coefficient (n_H) of 1.02 indicating kinetically the absence of heterogeneity of receptors and/or the absence of cooperativity between them (Fig. 1b, inset).

Time-course experiments (Fig.2) showed that specific binding increased monophasically and reached equilibrium within 15 min. Time to half-maximal bound was 186 ± 9 sec ([[^{3}H]prazosin] = 150.5 ± 3.2 pmole/l. n = 4). Because the Scatchard analysis of the saturation curve gave a straight line and the time-course curve was monophasic, the association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) for [^{3}H]prazosin were calculated according to the equation given in Materials and Methods. The equation plotted in the inset of Fig. 2 had a linear correlation coefficient of $r^2 = 0.97$. k_{+1} = 7.89×10^8 l./mole per min and $k_{-1} = 0.1160\ min^{-1}$. This gives a calculated $K_d = k_{-1}/k_{+1}$ = 147 pmole/l. (pk_d = 9,83). Thus, this plot did not indicate multiple rate constants.

The non-specific binding reached an apparent 'equilibrium' within the first minute and remained constant for the rest of the observed time.

Inhibition of specifically bound [^{3}H]prazosin by different adrenoceptor ligands

Inhibition of [^{3}H]prazosin binding to isolated myocardial cells by different adrenoceptor blockers displayed an order of potency: prazosin ⋙ phentolamine > yohimbine ⋙ propranolol (Fig. 3, Table 1), corresponding to an α_1-adrenoceptor subtype. Inhibition of [^{3}H]prazosin binding by adrenergic agonists also displayed an α-adrenoceptor order of potency: adrenaline > noradrenaline = phenylephrine > isoprenaline. Adrenaline was *ca* 100 times more potent than the β-adrenoceptor agonist isoprenaline (Fig. 4, Table 1).

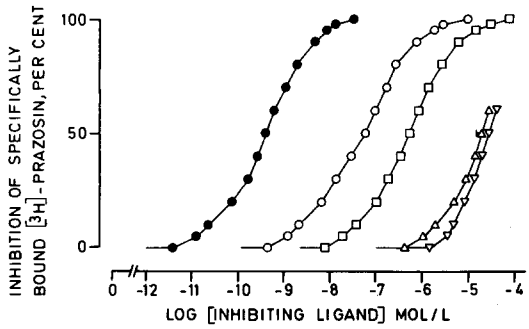

Fig. 3. Inhibition of specific [^{3}H]prazosin binding (see Materials and Methods) to isolated myocardial cells by increasing amounts of different adrenoceptor blockers: prazosin (●), phentolamine (○), yohimbine (□), (−)-propranolol (△) and (±)-propranolol (▽). Incubations were carried out for 15 min at 31°. [^{3}H]-Prazosin was *ca* 300 pmole/l. S.E.M. (range when n = 2) is given at 50% inhibition where it exceeds the width of the symbols; n = 3–4, except for propranolol where n = 2. Ordinate: inhibition of specifically bound [^{3}H]prazosin (per cent); abscissa: log of molar concentration of inhibiting ligand

Heterogenous α_1-adrenergic ligand binding in myocardial cells

When the data from the inhibition curves were

Table 1. Order of potency expressed as pIC_{50} values (mean ± S.E.M., n = 3–5, unless otherwise stated, $pIC_{50} = -\log IC_{50}$) for different adrenoceptor ligands determined by the inhibition of specifically bound [^{3}H]prazosin

	pIC_{50}	Potency ratio
Receptor blockers		
Prazosin	9.39 ± 0.08	1.0
Prazosin + propranolol	9.44 ± 0.04	
Trifluoperazine	7.21 ± 0.05 (range, n = 2)	6.6×10^{-3}
Phentolamine	7.24 ± 0.05	7.1×10^{-3}
Yohimbine	6.27 ± 0.09	7.6×10^{-4}
(−)-Propranolol	4.71 ± 0.07 (range, n = 2)	2.1×10^{-5}
(±)-Propranolol	4.55 ± 0.03 (range, n = 2)	1.4×10^{-5}
Agonists		
(−)-Adrenaline	5.51 ± 0.06	1.0
(−)-Adrenaline + propranolol	5.44 ± 0.03	
(−)-Noradrenaline	5.20 ± 0.06	0.5
(−)-Noradrenaline + propranolol	4.91 ± 0.04	0.25
(−)-Phenylephrine	5.13 ± 0.13	0.4
(−)-Phenylephrine + propranolol	4.81 ± 0.08	0.2
(−)-Isoprenaline	3.46 ± 0.07	8.9×10^{-3}

[[^{3}H]prazosin] ≈ 300 pmole/l. In some experiments 2×10^{-6} mole/l. (±)-propranolol was added 5 min before the competing ligands.

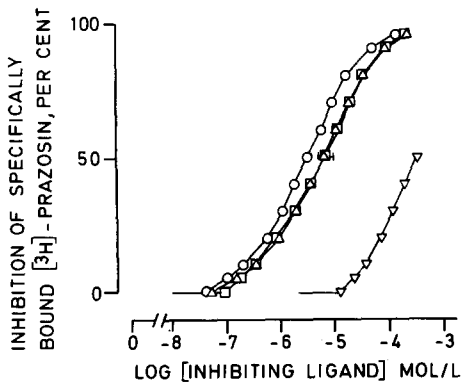

Fig. 4. Inhibition of specific [^{3}H]prazosin binding (see Materials and Methods) to isolated myocardial cells by increasing amounts of different adrenoceptor agonists: (−)-adrenaline (○), (−)-noradrenaline (△), (−)-phenylephrine (□) and (−)-isoprenaline (▽). Incubations were carried out for 15 min at 31°. [[^{3}H]Prazosin] was *ca* 300 pmole/l. S.E.M. is given at 50% inhibition where it exceeds the width of the symbols; n = 3–4. Coordinates as in Fig. 3.

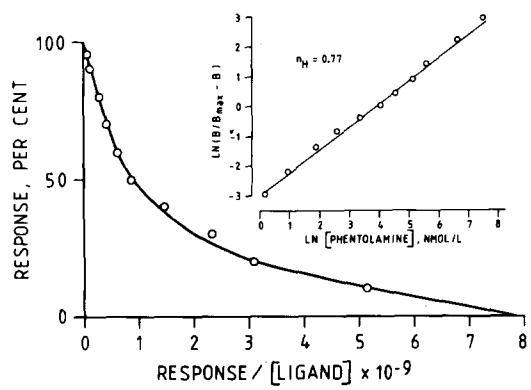

Fig. 6. Hofstee analysis (see Materials and Methods and legend to Fig. 5) of the inhibition of specifically bound [^{3}H] prazosin by phentolamine. Coordinates as in Fig. 5. The inset shows the Hill analysis of the inhibition data.

subjected to Hofstee analysis, they yielded curvilinear graphs (Figs. 5, 6 and 7a), indicating more than one binding component. Computer-assisted decomposition according to Minneman *et al.* [29] gave two binding populations (Figs. 5, 6 and 7a). In Table 2 is shown the estimated pK_d values and percentages for the two binding components of the receptor population obtained from the Hofstee plots. The different ligands showed different K_d ratios between the two components of binding, giving them different profiles. Both binding components were of the α_1-subtype of adrenoceptors according to the order of potency of blockers. At the higher affinity sites, the order of potency of agonists was adrenaline ≈ noradrenaline > phenylephrine, while at the lower affinity sites the order of potency was adrenaline ≈ noradrenaline ≈ phenylephrine (Table 2).

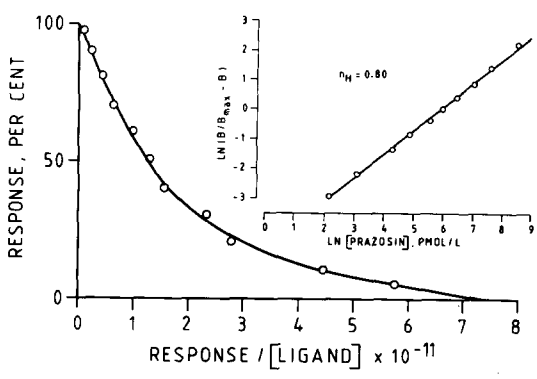

Fig. 5. Hofstee analysis (see Materials and Methods) of the inhibition of specifically bound [^{3}H]prazosin by prazosin. Ordinate: response (in per cent) expressed as increasing inhibition of radioligand-binding; abscissa: response (as defined for the ordinate) divided by the concentration of the inhibiting ligand giving this response. The inset shows the Hill analysis of the inhibition data.

As discussed by Minneman *et al.* [29], it is only an assumption that there are two, and not more, different types of binding sites when the Hofstee plot is curvilinear. These authors state, however, that this assumption can be validated in different ways. One reason why there may be only two types is that all ligands giving curvilinear Hofstee plots, except yohimbine, showed essentially constant percentages of the two subtypes of binding sites in the myocardial cells (Table 2). Yohimbine, however, detected apparently only *ca* 9% of the binding sites as high affinity sites (Table 2).

When the data were subjected to Hill analyses, they consistently showed Hill constants (n_H) in the range 0.75–0.85 (Fig. 5, inset and Fig. 6, inset) indicating either more than one type of binding sites or negative cooperative interactions.

Hofstee analysis of the inhibition of [^{3}H]prazosin by unlabelled prazosin also yielded a curvilinear graph, indicating two binding components (Table 2). In the Hill analysis, n_H was found to be 0.80. This indicated differences between the labelled and unlabelled forms of prazosin with regard to selectivity between apparent subgroups of α_1-adrenergic ligand binding sites, since the Scatchard plot and the Hill plot of the saturation experiments with the radioligand indicated one binding component only. As this difference between the labelled and unlabelled forms of prazosin was rather surprising, the saturation experiments were repeated with a longer incubation time (30 min). Neither in this situation could any indication of two binding components for [^{3}H]-prazosin be observed in Scatchard analysis [apparent K_d = 151.6 ± 17.1 pmole/l. (pK_d = 9.82), n = 4] nor in Hill analysis (n_H = 1.01). This difference between the labelled and unlabelled forms of prazosin was probably not a methodological artefact because also an unlabelled agent, trifluoperazine, detected only one binding component in inhibition experiments (see below).

Non-selective inhibition of specific [^{3}H]*prazosin binding by trifluoperazine*

Because phenothiazines are known to have α-adrenergic blocking properties and because pheno-

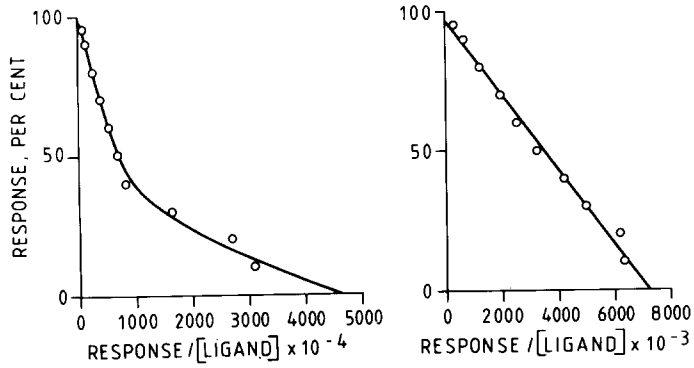

Fig. 7. Hofstee analysis (see Materials and Methods and legend to Fig. 5) of the inhibition of specifically bound [^{3}H]prazosin by phenylephrine in the absence (left panel) and presence (right panel) of 2 × 10^{-6} mole/l. propranolol. Coordinates as in Fig. 5.

thiazine compounds apparently can interfere with α-adrenoceptor binding in other tissues [2, 32], we also tested if trifluoperazine could interfere with α-adrenoceptor binding in heart cells. The inhibition of [^{3}H]prazosin by increasing doses of trifluoperazine is shown in Fig. 8. For comparison, the inhibition curves for prazosin and phentolamine are also shown in this plot. The K_d value for trifluoperazine was rather similar to the average K_d value for phentolamine (pK_d = 7,67 ± 0.06 and pK_d = 7.74 ± 0.02, respectively). In Fig. 9 is shown the Hofstee analysis of the trifluoperazine inhibition data. These data yielded a straight line accounting for the whole receptor population. Also Hill analysis (Fig. 9, inset) gave a straight line with a Hill constant (n_H) of 1.07. Both these graphs thus indicated that trifluoperazine was non-selective with respect to apparent subgroups of α_1-adrenergic ligand binding sites. The trifluoperazine interaction was competitive in nature and was rapidly reversed upon addition of [^{3}H]prazosin (data not shown). The pK_d value for trifluoperazine calculated from the shift of the saturation curve for [^{3}H] prazosin was 7.72 [33].

Inhibition of specifically bound [^{3}H]prazosin by different α-adrenoceptor ligands in the presence of propranolol

Functional studies of the α-adrenergic inotropic effects were performed in the presence of the β-adrenoceptor blocker propranolol [22]. Inhibition studies of the binding characteristics were therefore also performed in the presence of 2 × 10^{-6} mole/l. (±)-propranolol. The cells were preincubated for 5 min in the presence of propranolol before the addition of α-adrenoceptor ligands. The same order of potency of the agonists was observed in the absence and presence of propranolol. However, noradrenaline and phenylephrine were slightly, but significantly, less potent in the presence compared to the absence of propranolol (Table 1). Adrenaline, on the other hand, was apparently not influenced by propranolol (Table 1). Neither was the binding of the blocker prazosin (Table 1).

Hofstee analyses also revealed two binding components in this situation (Table 2), except for phenylephrine where only one component could be detected with a pK_d value not different from the

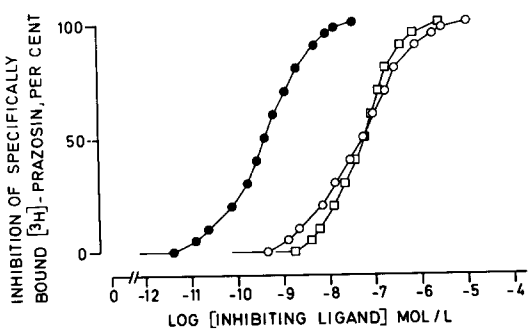

Fig. 8. Inhibition of specific binding of [^{3}H]prazosin (see Materials and Methods) to isolated myocardial cells by increasing amounts of trifluoperazine (□). The inhibition curves for prazosin (●) and phentolamine (○) are shown for comparison. Incubations were carried out for 15 min at 31°. [[^{3}H]Prazosin] was *ca* 300 pmole/l. Coordinates as in Fig. 3.

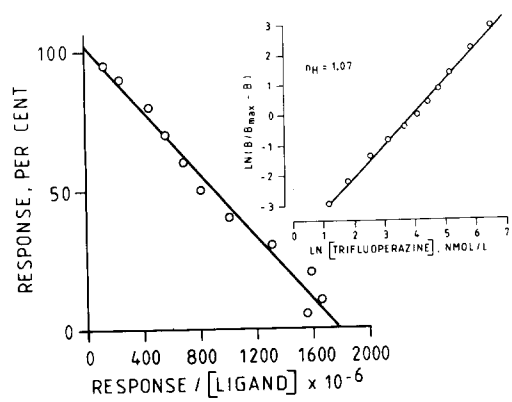

Fig. 9. Hofstee analysis (see Materials and Methods and legend to Fig. 5) of the inhibition of specifically bound [^{3}H]-prazosin by trifluoperazine. Coordinates as in Fig. 5. The inset shows the Hill analysis of the inhibition data.

Table 2. pK_d values and percentages of receptor population for the two binding components appearing in the Hofstee analyses of data obtained by the inhibition of specifically bound [^{3}H]prazosin by different α-adrenoceptor ligands

	pK_{d_1}	% of population	pK_{d_2}	% of population	Ratio pK_{d_2}/pK_{d_1}
Receptor blockers					
Prazosin	10.98 ± 0.13	22.5 ± 6.3	9.49 ± 0.07	77.5 ± 6.3	30.9
Prazosin + propranolol	10.81 ± 0.12	24.8 ± 1.9	9.38 ± 0.13	75.2 ± 1.9	26.9
Phentolamine	9.11 ± 0.42	23.0 ± 8.7	7.42 ± 0.23	77.0 ± 8.7	49.0
Yohimbine	8.11 ± 0.42	9.3 ± 3.5	6.38 ± 0.06	92.7 ± 3.5	53.7
		(Mean ± S.E.M., 19.9 ± 3.6, n =4)		(Mean ± S.E.M., 80.1 ± 3.6, n = 4)	
Agonists					
(−)-Adrenaline	6.95 ± 0.12	18.2 ± 2.9	5.48 ± 0.04	81.8 ± 2.9	29.5
(−)-Adrenaline + propranolol	6.89 ± 0.18	25.8 ± 4.1	5.43 ± 0.04	74.2 ± 4.1	28.8
(−)-Noradrenaline	7.23 ± 0.28	15.3 ± 0.5	5.40 ± 0.12	84.7 ± 0.5	67.6
(−)-Noradrenaline + propranolol	6.68 ± 0.35	20.2 ± 8.7	4.99 ± 0.18	79.8 ± 8.7	49.0
(−)-Phenylephrine	6.43 ± 0.12	23.8 ± 9.1	5.21 ± 0.41	76.2 ± 9.1	16.7
(−)-Phenylephrine + propranolol		(Mean ± S.E.M., 20.7 ± 1.9, n = 5)	5.11 ± 0.09	99.8 ± 0.5 (Mean ± S.E.M., 79.3 ± 1.9, n = 5, phenylephrine + propranolol not included)	—

In some experiments 2×10^{-6} mole/l. (±)-propranolol was added 5 min before the competing ligands.

pK_{d_2} value observed in the absence of propranolol. This component apparently accounted for 100% of the receptor population (Fig. 7b). Hill analyses also showed Hill constants (n_H) of 0.75–0.85, except for phenylephrine where $n_H = 0.97$ (not shown).

The concentration of (±)-propranolol used did not apparently interfere with the amount of [^{3}H]-prazosin bound (Fig. 3). However, when calculating the apparent affinities of the different inhibitors in the presence of propranolol, the equation developed by Cheng and Prusoff [30] for the presence of a constant amount of a third competitive substance was applied. The K_d value for (±)-propranolol obtained from the inhibition curve (calculated from IC_{10}) (Fig. 3) was used in these calculations.

DISCUSSION

Myocardial cells isolated from adult rat heart turned out to be a suitable preparation for ligand binding studies using [^{3}H]prazosin as the radiolabelled ligand. The apparent low non-specific binding and the rapid specific binding made this ligand especially suitable. Isolated myocardial cells appeared to have specific binding sites of high affinity and limited capacity, for α-adrenergic ligands. The binding of [^{3}H]prazosin could be competitively inhibited by unlabelled ligands. Inhibition experiments with different receptor blockers displayed the expected order of potency for the α_1-subtype of α-adrenoceptors.

Non-specific binding and intact cells

The apparent non-specific binding of [^{3}H]prazosin was surprisingly low and appeared to occur rapidly: after 1 min of incubation this binding had already reached 'equilibrium'. The apparent low non-specific binding was surprising because [^{3}H]prazosin is a lipophilic substance at the pH used, and because another lipophilic substance, [^{3}H]DHE, exhibited a high and variable non-specific binding (unpublished observations). The observed differences in nonspecific binding may reflect different distributions of the two substances in the cells. As the washing procedure was rather fast (completed within 15–20 sec), [^{3}H]-prazosin may be distributed to compartments from which it can easily be removed while [^{3}H]DHE may be distributed to compartments less accessible to washing. The non-specific binding of [^{3}H]DHE occurred rather slowly and 'equilibrium' was not always reached during the observed time period (20 min), in contrast to the specific binding which reached equilibrium within 10–15 min (unpublished observations). Thus, lipophilicity *per se* may not be the only factor determining the distribution patterns of these substances.

Subtypes of α_1-adrenoceptors

Inhibition of specific [^{3}H]prazosin binding by different ligands indicated heterogenous α-adrenoceptor binding sites in isolated myocardial cells. Hofstee analyses showed curvilinear graphs and Hill analyses showed $n_H = 0.75$–0.85 except for trifluoperazine. When the Hofstee plots are assumed to represent two binding sites, the percentages of these two binding components were estimated to be *ca* 20% for the higher affinity binding sites, and *ca* 80% for the lower affinity binding sites. Both binding sites were of the α_1-type according to the order of potency of receptor blockers. The order of potency of agonist was also characteristic of α-adrenoceptors.

The two apparent binding components may represent: (1) two different receptor types (subtypes of α_1-receptors); (2) same receptor type either (a) with different localization (and different accessibility for the ligands) or (b) with different affinity states; and (3) redistribution phenomena because different ligands may have different distribution barriers. At present, it is impossible to distinguish between these alternatives. Dissociation ('chase') experiments of different concentrations of [^{3}H]prazosin with the different unlabelled ligands at different concentrations may be an approach to decide which of the situation(s) is (are) the most likely. Inhibition experiments with unlabelled prazosin indicated two binding components (Table 2), in contrast to saturation and time-course experiments with [^{3}H]prazosin alone (Figs. 1 and 2). An ordinary 'chase' experiment of [^{3}H]prazosin with only a *high* concentration of unlabelled prazosin would hardly be sufficient to clarify these points as this experiment would only provide data on the binding characteristics of *labelled* ligand. An isotope effect of the tritium in [^{3}H] prazosin may be an alternative explanation of the observed differences between labelled and unlabelled prazosin.

Effects of propranolol upon inhibition of [^{3}H]prazosin binding by different ligands

Blocking the β-adrenoceptor stimulating component of noradrenaline and phenylephrine apparently reduced their potency at the α-adrenoceptor binding sites. In this situation, phenylephrine apparently recognized only one binding component with a $pK_d = 5.11 \pm 0.09$ (Table 2). This may not necessarily exclude the presence of two binding components, but may rather indicate that phenylephrine in this situation had affinity values for the two components that were not sufficiently different (less than 4–5-fold) to be detected as separate entities. Adrenaline, on the other hand, was apparently not influenced by the β-adrenoceptor blockade as also was the case for the α-receptor blocker, prazosin (Tables 1 and 2). Thus, concomitant stimulation of the β-adrenoceptor in some situations apparently influenced the properties of the α-adrenoceptor binding sites, thereby indicating a possible interaction between the two adrenergic receptors. The present investigation does not, however, give any clue to a mechanism of this possible interaction. Neither do we have any explanation why propranolol interfered differently with the binding of the three agonists. Further studies are needed to elucidate these questions.

Possible relations between receptors and function

As was mentioned in the Introduction, functional experiments in rat heart papillary muscles indicated differences in the α-adrenoceptor blocking pattern

of phentolamine and prazosin, respectively. This could suggest that the negative and positive parts of the α-adrenergic inotropic response might be mediated by different α-adrenergic receptors [22]. The present observation of two binding components would further be compatible with this possibility. Although it may be premature to speculate about the connection between the binding data and the functional experiments, some points are worth mentioning. Especially since the labelled ligand is a receptor blocker, one has to be cautious in assuming that all the specific binding sites represent physiological receptors. El Refai *et al.* [34], El Refai and Exton [35] and Geynet *et al.* [36] all found in rat liver that the agonists and blockers preferentially bind to different sites, making the 'blocker-binding' sites less likely to represent only the physiological receptors. There are, however, no corresponding observations in the heart. In the present experiments both agonists and blockers apparently showed both high and low affinity for the same binding site populations, respectively, as the percentages of the populations were about equal (*ca* 20 and 80%, respectively). Prazosin appeared also to be a very potent α-adrenergic antagonist in functional studies [22], being able to inhibit the α-adrenergic effects completely. Thus at least part of the binding components seems to be of physiological importance.

The inhibition constant, pA_2, of prazosin for the α-adrenergic positive inotropic effect in rat papillary muscle was 10.38–10.42 [22], which is more compatible with the higher affinity binding component [$pK_d = 10.81 \pm 0.12$ (Table 2)] than with the lower affinity binding component ($pK_d = 9.38 \pm 0.13$ (Table 2)] found in the present study. The latter might then mediate the negative inotropic component [22]. It is, however, not possible to explain the observed differences between the blocking pattern of prazosin and of phentolamine in functional studies (see Introduction) [22] in these terms because phentolamine has a relatively higher preference for the high affinity site than has prazosin, as is obvious from the K_{d_1}/K_{d_2} ratios of 49.0 and 30.9, respectively (Table 2). Thus, although the two binding components may serve different functions, it is impossible at present to relate the negative and the positive inotropic effects, respectively, only to one or the other binding type.

Concluding remarks

The present experiments showed that isolated myocardial cells possess specific high affinity binding sites for different α-adrenoceptor ligands. Analysis of the binding characteristics indicated two binding components of α_1-adrenoceptors. A resolution of the meaning of the two components is a further challenge. An increased affinity to the α-adrenoceptors by concomitant β-adrenoceptor stimulation indicated interaction between the two main groups of adrenergic receptors. As there is yet no known immediate biochemical event in the cell membrane coupled to α-adrenoceptor stimulation corresponding to the activation of the adenylate cyclase/cyclic AMP system after β-adrenoceptor stimulation, it is more difficult to relate binding studies to functional studies of the α-adrenergic system. By using intact cells suspended in a physiological salt solution, one may assume to have a preparation provided with endogenous levels of necessary components influencing the binding reaction, and to have a system that will reflect more closely ligand–receptor interactions in functional studies than a membrane preparation in an artificial buffer system will do. Isolated cells may also represent an approach to a better understanding of these mechanisms in humans, as Powell *et al.* [37] quite recently reported on preparations of isolated myocardial cells from human ventricular tissue.

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