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Identification and characterization of α_1-adrenergic receptors in human myometrium by [^{3}H]prazosin binding

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There is considerable evidence that myometrium exhibits contactile responses which are mediated by α-adrenergic stimulation [1, 2]. In this context, binding of the α-adrenergic antagonist [^{3}H]dihydroergocryptine, has been used to demonstrate the presence of, and to study α-adrenergic receptors in animal myometrium [3–6]. It has, however, become clear now that α-adrenergic receptors can be divided into two subclasses with different functional and pharmacological properties (i.e. the α_1-receptors mediating phosphatidylinositol turnover and α_2-receptors mediating adenylate cyclase inhibition [7–9]), and that [^{3}H]-dihydroergocryptine binding does not discriminate between these subclasses. Moreover, [^{3}H]dihydroergocryptine has also been shown to have a reasonably high affinity for dopamine and 5-hydroxytryptamine receptors in rat brain [10, 11], and the physiological significance of its binding sites in smooth muscle has been questioned [12].

During the past few years, several new radioligands have been developed and used to monitor and the presence of either α_1- or α_2-adrenergic receptors. They include the antagonists [^{3}H]prazosin for the labelling of α_1-receptors [13, 14] and [^{3}H]yohimbine for α_2-receptors [15]. In the present study, we have been for the first time able to directly demonstrate the presence of, and to characterize α_1-adrenergic receptors in myometrium membrane fractions using [^{3}H]prazosin binding.

Materials and methods

Materials. The following were obtained as generous gifts: (+)- and (−)-epinephrine bitartrate and (−)-isoproterenol bitartrate (Sterling-Winthrop); phentolamine hydrochloride (Ciba-Geigy); prazosin hydrochloride (Pfizer); phenoxybenzamine hydrochloride (Smith Kline & French); indoramine hydrochloride (Wyeth); yohimbine hydrochloride (Roussel); (−)-propranolol hydrochloride (Imperial Chemical Industries); and clonidine hydrochloride (Boehringer-Ingelheim). (—)-Norepinephrine tartrate, dopamine hydrochloride and (—)-phenylephrine hydrochloride were purchased from Sigma and pyrocatechol from Carbo Erba. [^{3}H]Prazosin (28 Ci/mmole) was from Amersham. All other chemicals and reagents were of the highest grade commercially available.

Source of tissue and preparation of membranes. Uteri were obtained under routine anesthesia from women undergoing abdominal or vaginal hysterectomy for various medical or surgical indications. All subsequent steps were performed at 0°. Samples were brought to the laboratory within 10 min, dissected free of endometrium and serosa. The myometrium obtained was chopped and homogenized in 5 mM Tris–HCl (pH 7.4)/2 mM $MgCl_2$ in a Virtis 45 homogenizer at 45,000 rpm for 5 min. The tissue/buffer ratio was 1/10 (w/v). The homogenate was then centrifuged in a Sorvall centrifuge at 1000 *g* for 10 min. The supernatant was removed and centrifuged at 5000 *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 40,000 *g* for 20 min. The final pellet was resuspended in 50 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$. (incubation buffer) and stored in the presence of glycerol (10% v/v) in liquid nitrogen at a protein concn of approximately 10 mg/ml. Protein concns were determined according to the method described by Lowry *et al.* [16].

Binding of [^{3}H]*prazosin.* Binding of [^{3}H]prazosin to human myometrial membranes was assayed by filtration on glass fiber filters. Membrane proteins (0.5 mg/ml) were incubated with the indicated concns of [^{3}H]prazosin (0.2–4 nM) for 10 min at 37° in incubation buffer in a final vol. of 500 μl. At the end of the incubation, the samples were diluted in 4 ml of ice-cold buffer, and filtered under reduced pressure through glass fiber filter discs (Whatman GF/F, 2.5 cm dia.). Filters were washed rapidly 4 times with 4 ml of ice-cold buffer, placed in 20 ml polyethylene scintillation vials with 1 ml of 0.1 N NaOH and 10 ml of scintillation fluid (Aqualuma from Lumac) and counted in a Packard liquid scintillation spectrometer. In all experiments, the amount of non-specific [^{3}H]prazosin binding was determined by incubation of membranes and [^{3}H]prazosin in the presence of 10 μM unlabelled phentolamine. Specific binding was obtained by substracting non-specific binding from total binding, and approximated 70% of total binding at 1 nM of tracer. In all figures and tables, bound [^{3}H]prazosin refers to specific binding as defined earlier.

Results and discussion

Binding of [^{3}H]prazosin to human myometrium membranes was a saturable process of high affinity. As shown in Fig. 1, specific binding reaches a plateau value upon increasing the free concn of tracer. Scatchard analysis [17] of the saturation binding data reveals a single class of binding sites (38 fmoles/mg protein) with an equilibrium dissociation constant of 0.73 nM for the myometrium membrane sample used in Fig. 1. The linearity of the Scatchard plot and the Hill coefficient (n_h) of 1.03 ($r = 0.993$) do not indicate meaningful cooperative interactions among the binding sites. The calculated number of binding sites varied from 8 to 69 fmoles/mg protein, depending on the uterus tested. The equilibrium dissociation constant for [^{3}H]prazosin binding (K_d) showed only little variability (mean ± S.E.M. = 0.7 ± 0.2 nM for the 43 uteri tested).

The binding of [^{3}H]prazosin is a rapid and reversible process. In the kinetic experiment shown in Fig. 2, membranes are incubated with [^{3}H]prazosin at 37°, and the amount of specifically bound tracer expressed as a function of the incubation time. Binding reaches equilibrium within 1 min, which is too fast for an accurate calculation of the association rate, and remains steady for at least 10 min. Dissociation of [^{3}H]prazosin from its binding sites was measured by adding an excess of phentolamine (10 μM) to the equilibrated mixture of tracer and myometrium membranes (arrow in Fig. 2). Dissociation at 37° was complete (99% displacement of original binding within 20 min), and followed first-order rate kinetics with a rate constant (k_{-1}) of 0.0032 sec^{-1}.

Adrenergic agonists competed with [^{3}H]prazosin for binding to the total amount of specific sites. Competition binding by (−)-epinephrine is shown in Fig. 3. The Hill plot of these data (Fig. 3 insert) is linear ($r = 0.997$) and has a slope of 1.1. This indicates that the agonist interaction with the prazosin binding sites also follows a simple mass action. Competition binding by various adrenergic agonists occurred with the order of potencies expected for an α_1-adrenergic receptor [12]: i.e. (−)-epinephrine > (−)-norepinephrine > (−)-phenylephrine > clonidine > (+)-

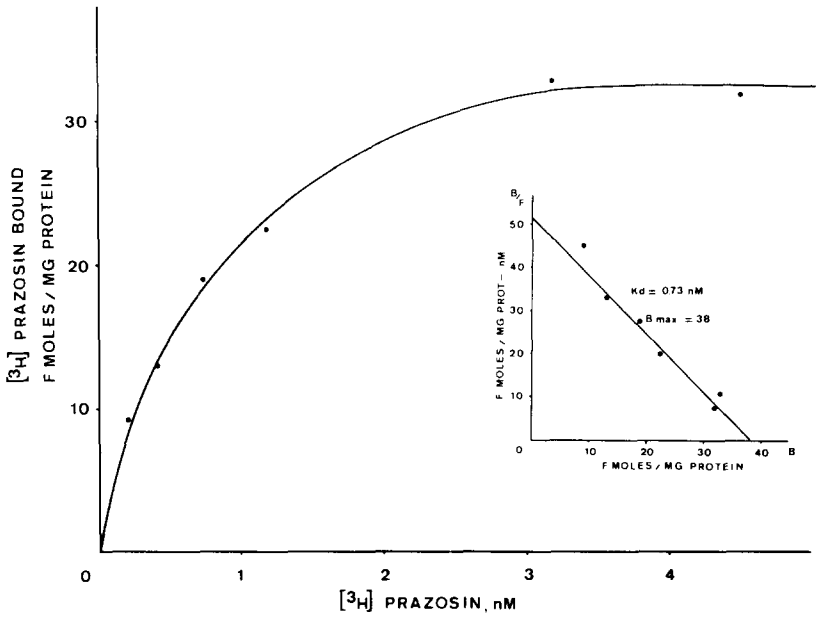

Fig. 1. [^{3}H]prazosin binding to human myometrial membranes. Human myometrial membranes were incubated with increasing concns of [^{3}H]prazosin (0.1–5 nM) for 10 min at 37°, and filtered. Non-specific binding measured in the presence of 10 μM unlabelled phentolamine. Data shown represent specific binding, i.e. total minus non-specific binding. Insert: Scatchard plot of the saturation binding data. The slope was determined by linear regression analysis. The total amount of binding sites was 38 fmoles/mg protein and the equilibrium dissociation constant for binding (K_d) was 0.73 nM.

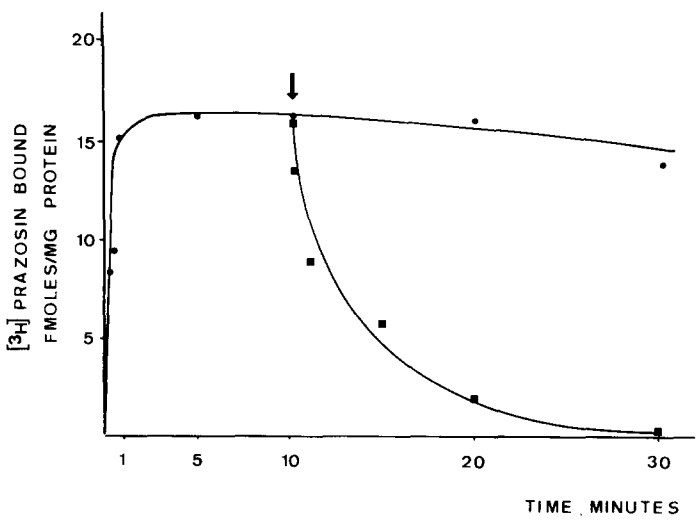

Fig. 2. Time-course of [^{3}H]prazosin binding. (●——●) Membranes were incubated with 4 nM of tracer at 37° for increasing periods of time (abscissa) and the amount of specific binding measured. The concn of tracer is well over that of the binding sites (0.04 nM), so that the concn of free tracer may be considered constant. (■——■) At the arrow, phentolamine was added to the incubation mixture (in 5% of the final vol.) at a final concn of 10 μM, and the incubation was continued.

epinephrine ≫(−)-isoproterenol (Table 1). The marked stereospecificity of the sites towards levoratary isomers is evidenced by the 50 times greater potency of (−)-epinephrine compared with its corresponding (+)-isomer to displace bound tracer, with K_i values (calculated from the concn of agonist causing 50% displacement of specific binding by the method of Cheng and Prusoff [18]) of 0.15 and 8.07 μM, respectively.

Adrenergic antagonists also competed with [^{3}H]prazosin for binding with the expected specificity: i.e. prazosin > phenoxybenzamine > indoramine > phentolamine ≫yohimbine (Table 1). From the α-adrenergic antagonists tested the α_1-selective drug prazosin has the highest affinity, and the α_2-selective drug yohimbine has the lowest affinity for the binding sites. In addition, the β-adrenergic antagonist (−)-propranolol, dopamine and the non-bioactive compound pyrocatechol did not show any measurable displacement of [^{3}H]prazosin binding at 0.1 mM.

These data clearly stress the α_1-adrenergic specificity of the [^{3}H]prazosin binding sites in human myometrium mem-

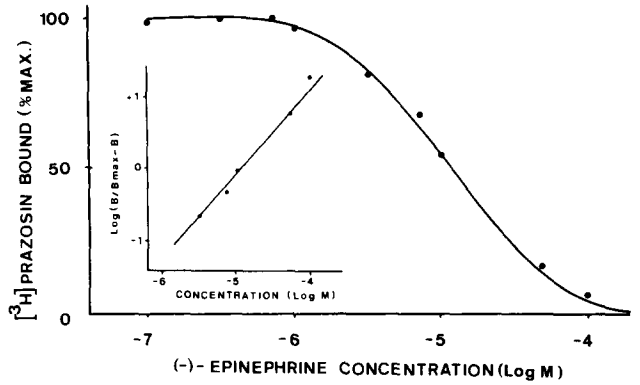

Fig. 3. Competition binding by (−)-epinephrine. Membranes were incubated with increasing concns of (−)-epinephrine (abscissa) in the presence of 4 nM [^{3}H]prazosin, and binding measured. Control (100%) binding was measured in the presence of buffer only. Insert: Hill plot of the competition binding data: B represents per cent binding of (−)-epinephrine (i.e. 100 − per cent [^{3}H]prazosin binding), and B max represents 100% binding. The slope was determined by linear regression analysis, and equalled 1.1 ($r = 0.997$).

Table 1. Agonist and antagonist K_i values for human myometrial [^{3}H]prazosin binding sites

Adrenergic drug added	K_i value (nM)
Agonists	
(−)-Epinephrine	230
(−)-Norepinephrine	700
Phenylephrine	1600
Clonidine	4300
(+)-Epinephrine	12,000
(−)-Isoproterenol	> 100,000
Antagonists	
Prazosin	0.22
Phenoxybenzamine	1.5
Indoramine	4.5
Phentolamine	4.9
Yohimbine	11,000
(−)-Propranolol	> 100,000

Membranes were incubated with increasing concns of the listed adrenergic drugs in the presence of 2.4–5.3 nM [^{3}H]prazosin. A typical example for (−)-epinephrine is shown in Fig. 3. The K_i values were calculated by the method of Cheng and Prusoff [18] from the drug concns causing 50% displacement of [^{3}H]prazosin binding.

branes. The presence of this receptor subclass has been previously suggested in animal uterine tissue using the radioligand [^{3}H]dihydroergocryptine [19]. However, this ligand has been shown to bind with uniform affinity to both α_1- and α_2-adrenergic receptors [4]. Accordingly, the characterization of the α_1-adrenergic receptors could only be performed indirectly by analysis of complex competititon binding curves using a limited number of ligands showing either a high α_1- or α_2-selectivity [19]. The affinities of less selective adrenergic ligands, including the catecholamines, could be determined for either receptor subclass using this technique.

As shown in this report, α_1-adrenergic receptors can be directly characterized in myometrial membranes using the specific α_1-adrenergic radioligand [^{3}H]prazosin. Compared to the previous approach, this allows a more precise determination of the α_1-adrenergic receptor number and of their agonist and antagonist binding properties. In conclusion, use of this radioligand might prove to be a powerful tool in distinguishing α_1-receptors from the total amount of α-adrenergic receptors. As a practical application, it becomes now possible to determine whether the reported influence of sex steroid hormones on the total α-adrenergic receptor population in animal uteri does also apply to the α_1-subpopulation.

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Fondation pour la Recherche en Endocrinologie Sexuelle et de la Reproduction Humaine
Service de Gynécologie et d'Obstétrique
Hôpital Universitaire Brugmann
Université Libre de Bruxelles
†*Laboratorium Chemie der Proteinen*
Vrije Universiteit Brussel
Belgium

SERGE P. BOTTARI*
GEORGES VAUQUELIN
JEAN PAUL LESCRAINIER
ETIENNE KAIVEZ
ALAIN VOKAER

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* To whom all correspondence should be addressed at: Fondation pour la Recherche en Endocrinologie Sexuelle et de la Reproduction Humaine, Service de Gynécologie et d'Obstétrique, Hôpital Universitaire Brugmann, Place Van Gehuchten, 4, 1020 Brussels, Belgium.

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Kinetics of binding of the semi-synthetic α-methyl-digitoxigenin-glucoside to cardiac (Na^+–K^+)ATPase

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3-α-Methyl-digitoxigenin-3-β-glucoside (methyl-dtg-gluc) is a semi-synthetic cardiac glycoside distinguished by two peculiar properties when compared to classic cardiac glycosides: firstly, a fast onset of the positive inotropic effect and a rapid reversibility even of toxic effects upon removal [1–3]; secondly, dose–response curves covering a wider concn range and reaching higher inotropic maxima [1, 2, 4]. In order to characterize the binding properties of methyl-dtg-gluc at the receptor level, equilibrium binding studies were reported with [^{3}H]methyl-dtg-gluc and [^{3}H]ouabain, which revealed that methyl-dtg-gluc binds to ouabain binding sites in cardiac membranes with an affinity more than 10-fold lower than ouabain [5]. Complementary to that, in the present study the kinetic features of the interaction between methyl-dtg-gluc and its binding site were examined, measuring the association and dissociation of [^{3}H]methyl-dtg-gluc with the (Na^+–K^+)ATPase (EC 3.6.1.3) present in a crude membrane suspension of guinea pig ventricular muscle. The results were compared with the kinetic data of [^{3}H]ouabain binding. Methyl-dtg-gluc associated more than 2 times faster, while its dissociation proceeded at least 30 times faster, its affinity thus being more than 10-fold lower. This extraordinarily high dissociation rate is in accordance with the rapid reversibility of inotropic and toxic effects induced by methyl-dtg-gluc.

Materials and methods

[^{3}H]Ouabain (14 Ci/mmole) was obtained from NEN (Dreieich, F.R.G.), [^{3}H]methyl-dtg-gluc (11.1 Ci/mmole) was a gift from Knoll AG (Ludwigshafen, F.R.G.) as well as unlabelled methyl-dtg-gluc. All other chemicals were purchased from E. Merck (Darmstadt, F.R.G.).

Preparation of cardiac membranes. Cardiac ventricles of guinea pigs (either sex, 300–500 g) were frozen and stored at −20°. The duration of storage at −20° up to several weeks was without influence on the experimental results. Compared with hearts not subjected to the freezing procedure the yield of binding sites was a little increased when frozen ventricles were used for the preparation. At a temperature of 4° the ventricles were thawed, minced and homogenized (Waring blender, low speed, 30 sec) in a 0.32 M sucrose solution (20 ml/g wet wt). After treatment with six strokes in a Potter–Elvehjem glass homogenizer with a motor-driven Teflon pestle, the homogenate was centrifuged (10 min at 2000 *g*); the supernatant was recentrifuged (18 min at 30,000 *g*). The pellets were resuspended in 50 mM Tris–HCl adjusted to pH 7.4 (4 ml/g wet wt), frozen in liquid nitrogen and stored at −20° until the day of experiment.

Binding experiments. The equilibrium binding experiments have been described in detail [5]. Similarly, the kinetic experiments were performed at 37° in a medium (total vol. 45 ml) consisting of the membrane suspension (final concn of binding sites about 5 pmoles/ml), of NaCl (80 mM), $MgCl_2$ (16 mM), Tris–HCl (50 mM, final pH 7.3) and of the tritium-labelled cardiac glycoside (about 10 nM).

After a preincubation period of 30 min, ATP was added (final concn 2.5 mM) to initiate specific binding. After 45 min, when binding had attained an equilibrium level, unlabelled ouabain was added in excess (6.3×10^{-5} M) and the dissociation of the radioactive ligand was observed ("chase experiment"). The samples taken from the magnetically stirred medium had a vol. of 1 ml and were filtered under suction through Whatman GF/C filters and rinsed with 2 × 5 ml of ice-cold distilled water within 15 sec. After addition of Soluene 350® and Dimilume 30® (Packard, Frankfurt, F.R.G.) the filter-bound radioactivity was counted.

The unspecific binding was identical within the limits of experimental scattering, whether determined as the ATP-independent binding before addition of ATP or as the "non-displaceable" binding in the presence of the excess of ouabain at the end of the dissociation reaction (Fig. 1); specific binding was the difference between the total and the unspecific binding.

The association rate constant (k_{+1}) was calculated using the equation $d[RL]/dt = k_{+1} \times [L] \times [R]$, which describes the initial association velocity; [L] was considered equal to