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Pharmacological pleiotropism of the human recombinant α_{1A}-adrenoceptor: implications for α_1-adrenoceptor classification

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1 Three fully-defined α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) have been established in pharmacological and molecular studies. A fourth α_1-adrenoceptor, the putative α_{1L}-adrenoceptor, has been defined in functional but not molecular studies, and has been proposed to mediate contraction of human lower urinary tract tissues; its relationship to the three fully characterized α_1-adrenoceptors is not known.

2 In the present study, binding affinities were estimated by displacement of [^{3}H]-prazosin in membrane homogenates of Chinese hamster ovary (CHO-K1) cells stably expressing the human α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors and were compared with affinity estimates obtained functionally in identical cells by measuring inhibition of noradrenaline (NA)-stimulated accumulation of [^{3}H]-inositol phosphates.

3 For the α_{1A}-adrenoceptor, binding studies revealed a pharmacological profile typical for the classically defined α_{1A}-adrenoceptor, such that prazosin, RS-17053, WB 4101, 5-methylurapidil, Rec 15/2739 and S-niguldipine all displayed subnanomolar affinity. A different profile of affinity estimates was obtained in inositol phosphates accumulation studies: prazosin, WB 4101, 5-methylurapidil, RS-17053 and S-niguldipine showed 10 to 40 fold lower affinity than in membrane binding. However, affinity estimates were not 'frameshifted', as tamsulosin, indoramin and Rec 15/2739 yielded similar, high affinity estimates in binding and functional assays.

4 In contrast, results from human α_{1B}- and α_{1D}-adrenoceptors expressed in CHO-K1 cells gave antagonist affinity profiles in binding and functional assays that were essentially identical.

5 A concordance of affinity estimates from the functional (inositol phosphates accumulation) studies of the α_{1A}-adrenoceptor in CHO-K1 cells was found with estimates published recently from contractile studies in human lower urinary tract tissues (putative α_{1L}-adrenoceptor). These data show that upon functional pharmacological analysis, the cloned α_{1A}-adrenoceptor displays pharmacological recognition properties consistent with those of the putative α_{1L}-adrenoceptor. Why this profile differs from that obtained in membrane binding, and whether it explains the α_{1L}-adrenoceptor pharmacology observed in many native tissues, requires further investigation.

Keywords: α_1-Adrenoceptors; noradrenaline; prazosin; inositol phosphates; radioligand binding; second messenger; RS-17053; tamsulosin

Introduction

Pharmacological and molecular cloning studies have established operational and structural heterogeneity among α_1-adrenoceptors (Minneman, 1988; Ford *et al*., 1994; Bylund *et al*., 1994). The current classification recognizes the existence of three α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) all of which meet well established criteria (i.e., sequence, second messenger and pharmacological profile: Hieble *et al*., 1995).

A fourth α_1-adrenoceptor, the so-called α_{1L}-adrenoceptor, has been postulated (Holck *et al*., 1983; Flavahan & Van-Houtte, 1986; Muramatsu, 1992). This postulate is based on pharmacological criteria only as the cDNA for a novel α_1-adrenoceptor has not been isolated. The present paper throws new light on the nature of the α_{1L}-adrenoceptor and provides evidence that this receptor may not derive from a distinct gene. Instead, the α_{1L}-adrenoceptor may represent a particular, energetically favourable conformational state of the α_{1A}-adrenoceptor.

Data from our laboratory have established previously that the functional pharmacological properties of the putative α_{1L}-adrenoceptor in anococcygeus muscle of rat and human lower urinary tract smooth muscle are closest to those of the α_{1A}-adrenoceptor (Ford *et al*., 1993; 1996). For this reason, we conducted a thorough characterization of the cloned human α_{1A}-, α_{1B} and α_{1D}-adrenoceptors (expressed in Chinese hamster ovary cells; CHO-K1) with both functional (noradrenaline (NA)-stimulated second messenger generation) and ligand binding measurements, in order to assess their pharmacological resemblance with native tissue characterizations. CHO-K1 cells lack endogenous adrenoceptors and thus offer a unique opportunity to study the gene product or products resulting from transfection with the cDNAs for α_1-adrenoceptors of man.

The important finding from this study was that functional estimates of antagonist affinity for the α_{1A}-adrenoceptor correlated closely with those established for the α_{1L}-adrenoceptor (Muramatsu, 1992; Ford *et al*., 1993; 1996), whereas ligand binding studies yielded affinity estimates which are fully consistent with the documented α_{1A}-adrenoceptor (Hieble *et al*., 1995). Thus, contingent upon the method of study, and presumably on certain hitherto undetermined environmental factors, the same α_{1A}-adrenoceptor gene product can display more than one pharmacological phenotype. In global terms, these findings affect the interpretation of pharmacological data for the classification of receptors.

Methods

Cloning and expression of human α_1-adrenoceptor subtypes

Full length coding regions of the human α_{1a}-, α_{1b}- and α_{1d}-adrenoceptor genes were cloned by PCR amplification with subtype-specific primers on cDNAs from human prostate,

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aorta and hippocampus, respectively. The amplified products were cloned into the eukaryotic expression vector, pSW104, a derivative of pCD-SRα (Takebe *et al*., 1988). Chinese hamster ovary (CHO-K1) cells stably expressing the α_1-adrenoceptor subtypes were obtained by co-transfection of the receptor cDNAs with the plasmid pSV2neo using lipofection (Flegner *et al*., 1987). Stable clones were initially selected for resistance to G418 (500 mg ml^{-1}) and then maintained in Ham's F-12 nutrient media supplemented with 10% foetal bovine serum, geneticin (150 μg ml^{-1}), penicillin/streptomycin (30 u ml^{-1}; 30 mg ml^{-1}), G418 (250 mg ml^{-1}) at 37°C in 7% CO_2.

Radioligand binding studies

Estimates of affinity (pK_i) at α_1-adrenoceptors for competitive α_1-adrenoceptor antagonists were made from competition curves (by use of 10 concentrations of displacing agent) in membrane homogenates of Chinese hamster ovary (CHO-K1) cells transfected stably to express human cloned α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors, as described previously (Blue *et al*., 1995). [^{3}H]-prazosin (0.1 nM; specific activity 82 Ci $mmol^{-1}$) was used to label α_1-adrenoceptors and phentolamine (10 μM) was employed to determine specific binding. All equilibrations were carried out for 60 min at room temperature (20°C). Concentrations of competing agent producing 50% displacement of [^{3}H]-prazosin (IC_{50}) were interpolated by use of non-linear iterative curve-fitting methodologies and converted into estimates of affinity (pK_i) according to the equation of Cheng and Prusoff (1973). Estimates of K_D for [^{3}H]-prazosin were made by saturation analysis with 12 concentrations of ligand (1 pM – 5 nM).

Inositol phosphates accumulation

The method used was a modification of well-established procedures (Brown *et al*., 1984). Briefly, cells were washed with phosphate buffered saline (PBS) and incubated in 15 ml inositol free Ham's F-12, containing 10% dialyzed foetal bovine serum and 25 μCi [^{3}H]-*myo*-inositol, overnight. Following the incorporation of inositol, media was aspirated and the cells washed with PBS to remove unincorporated [^{3}H]-*myo*-inositol. PBS containing EDTA (ethylene diamine tetraacetic acid; 30 μM for 5 – 10 min at 37°C) was used to dissociate cells from the flask. The cell suspension was spun for 5 min at $500 \times g$ at 37°C and washed 3 times in PBS. The cells were resuspended in inositol-free Ham's buffer to $\sim 5 \times 10^6$ cells ml^{-1}. Reactions were performed in triplicate tubes containing 300 μl final reaction volume. Cell suspension (240 μl) was added for pre-equilibration to 30 μl antagonist or vehicle at 37°C for 20 min. The reaction was initiated by the addition of 30 μl agonist or vehicle, containing LiCl (final concentration 10 mM). Tubes were then gently mixed and placed in a 37°C bath for 10 min. Reactions were terminated by the addition of 50 μl ice-cold perchloric acid (20%). Tubes were allowed to sit in an ice-water bath for 20 min and samples were then neutralized with 160 μl 1 M KOH, vortex-mixed and spun at $1000 \times g$ at 4°C for 10 min. Samples were then gently diluted with the addition of 2 ml Tris-HCl (50 mM, pH 7.5) and decanted onto disposable columns containing 1 ml Dowex AG 1X8, chloride form (1 : 1, w/v) slurry which had been washed with 5 ml distilled H_2O. Columns were then washed with 20 ml distilled H_2O and the eluate discarded. [^{3}H]-inositol phosphates (InsPs) were eluted with 3 ml HCl (1 M) into scintillation vials containing 15 ml Ready-Safe liquid scintillation cocktail.

Accumulated [^{3}H]-InsPs were measured by liquid scintillation spectroscopy by a Packard 1900TR and expressed as d.p.m. by use standard ^{3}H window settings. The d.p.m. values were imported into Microsoft Excel for determination of mean and standard deviation values for each triplicate determination. Iterative nonlinear curve-fitting methods by Kaleidagraph software were used to fit data to the general logistic functions: $E = \text{basal} + E_m$. $A^{nH}/(A^{nH} + [A]_{50}{}^{nH})$ for agonist stimulation curves; $E = \text{basal} + E_m - (E_m . B^{nH}/(B^{nH} + [B]_{50}{}^{nH}))$ for antagonist inhibition curves. $[A]_{50}$ or $[B]_{50}$ values, maxima (E_m) and Hill slopes (n_H) for each curve were estimated by use of this software. Affinity values of test substances (pK_b) were calculated according to Leff and Dougall (1993), such that $K_b = [B]_{50}/((2 + ([A]/[A]_{50})^{nH})^{1/nH} - 1)$. In the case of some antagonists (prazosin and RS-17053), the surmountability of antagonism was investigated by constructing E/[A] curves to agonist in the absence and presence of fixed antagonist concentrations for construction of Schild regression analyses with affinity estimates determined according to the relationship,

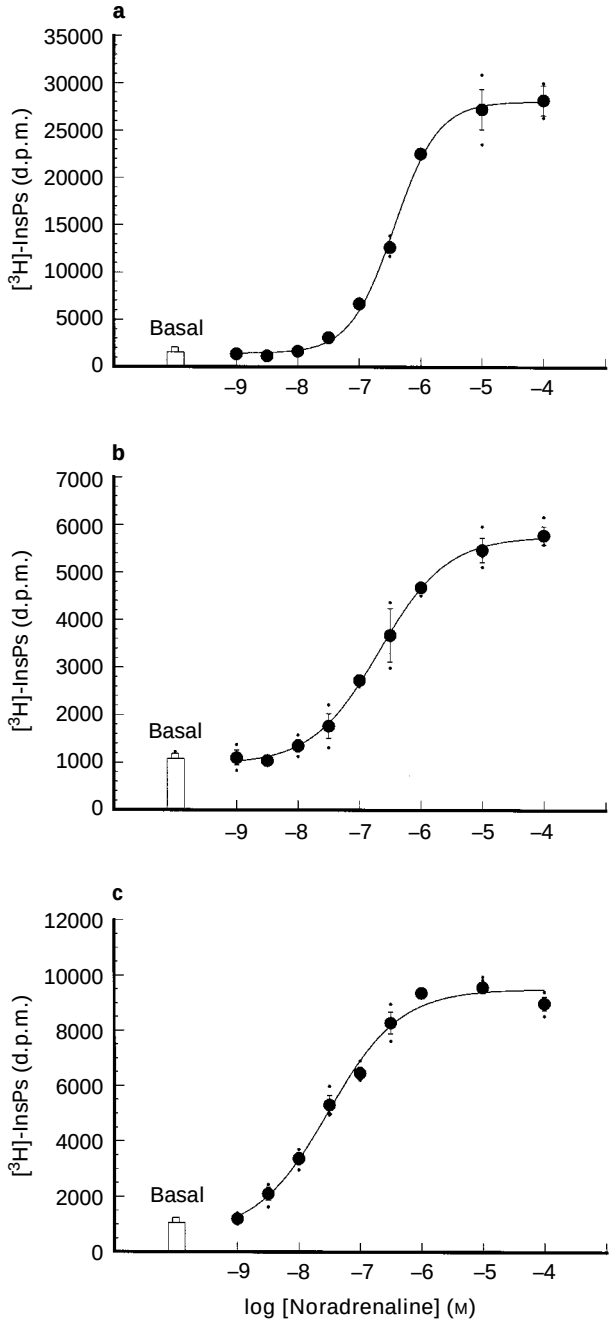

Figure 1 Noradrenaline (NA)-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human cloned (a) α_{1A}- (b), α_{1B}- and (c) α_{1D}-adrenoceptors. Data shown are from single experiments, which were performed in triplicate. Within-experiment means and standard deviations are shown (large symbols), as are individual data points, through which non-linear regressions were constructed. Each experiment was repeated, with the number of experiments shown in Table 1. Basal levels of [^{3}H]-InsPs accumulation were similar in the three cell populations; maximal stimulations in response to NA differed, with greatest stimulation observed in CHO-K1 cells expressing α_{1A}-adrenoceptors (see Table 1).

$pK_b = -\log [B] + \log [r-1]$ (where r is the ratio of agonist $[A]_{50}$ estimates in the presence versus the absence of antagonist, B), when regression slopes were not different significantly from 1. This latter method also provided an alternative affinity estimate for those antagonists (e.g., *S*-niguldipine and tamsulosin) that produced unusually steep inhibition curves ($n_H > 1.5$), due presumably to inadequate equilibration time or loss of chemical at lower concentrations (degradation or adsorption into materials).

Pharmacological terms and definitions used throughout this manuscript are in accordance with recently published IUPHAR guidelines (Jenkinson *et al*., 1995).

Materials

Ham's F-12 nutrient media, phosphate buffered saline, geneticin (G418), fetal bovine serum (qualified and dialyzed), penicillin/streptomycin and versene (EDTA) were obtained from Gibco (Gaithersburg, MD). *Myo*-[2-^{3}H]-inositol (10–20 Ci mmol^{-1}) was obtained from Amersham (Arlington Heights, IL). Disposable plastic columns (CC-09-M) were obtained from E & K Scientific Products (Saratoga, CA). *S*(+)-niguldipine, prazosin, WB 4101 ((2,6-dimethoxypheno xyethyl)aminomethyl-1,4-benzodioxane hydrochloride), oxymetazoline and 5-methylurapidil were obtained from Research Biochemicals International (Natick, MA). Noradrenaline and bulk chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO). RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl] - 5-chloro-α,α-dimethyl-1H-indole-3-ethanamine hydrochloride), Rec 15/2739 (SB 216469; 8-3-[4-(2-methoxyphenyl) -1- piperazinyl] - propylcarbamoyl-3-methyl-4-oxo-2-phenyl-4H-1-benzopyran dihydrochloride), NS 49 ((**R**)-(−)-3′-(2-amino-1-hydroxyethyl)-4″-fluoromethane sulphonanilide hydrochloride), SDZ NVI 085 ((**R**,**S**) 3,4,4a5,10,10a-hexahydro-6-methoxy-4-methyl-9-methylthio-2H-naphth [2,3-b]-1,4-oxazine hydrochloride), A-61603 (N-[5-(4,5-dihydro-1H-imidazol-2yl) -2 - hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide) and other compounds were synthesized in the Department of Chemistry, Neurobiology Unit, Roche Bioscience (Palo Alto, CA). Kaleidagraph Software was purchased from Synergy Software (Reading, PA). Ready-Safe liquid scintillation cocktail was purchased from Baxter Scientific (McGraw Park, IL).

Results

Second messenger studies, measuring [^{3}H]-InsPs accumulation, were done in intact CHO-K1 cells expressing cloned human α_{1A}-, α_{1B}- or α_{1D}-adrenoceptors. Noradrenaline (NA, 1 nM to 10 μM) elicited robust concentration-dependent increases in [^{3}H]-InsPs accumulation yielding maximal stimulations of 10, 3.7 and 4.1 fold, respectively, above basal [^{3}H]-InsPs levels (Figure 1). Potency estimates ($p[A]_{50}$) for NA (Table 1) suggested the presence of receptor reserve for this agonist when compared with affinity estimates obtained previously (Minneman *et al*., 1994; Blue *et al*., 1995). The results also confirm the higher potency of NA for the α_{1D}-adrenoceptor, for which it has been demonstrated to display an approximately 10 fold higher affinity (Forray *et al*., 1994; Minneman *et al*., 1994; Testa *et al*., 1995; Tseng-Crank *et al*., 1995; Shibata *et al*., 1995). Phenethylamine and imidazoline agonists, with varying selectivities for the α_{1A}-adrenoceptor (see Minneman *et al*., 1994; Blue *et al*., 1995) were also examined. Table 1 illustrates the potencies ($p[A]_{50}$) and intrinsic activities of the agonists, relative to NA (which was assayed in all experiments). The imidazoline agonists, A-61603 and oxymetazoline, displayed highest potency, although the latter compound behaved as a partial agonist. All agonists displayed potencies and intrinsic activities within the range expected for a functional α_{1A}-adrenoceptor bioassay (Minneman *et al*., 1994; Blue *et al*., 1995).

Figure 2 shows the effect of several antagonists on the generation of [^{3}H]-InsPs in CHO-K1 cells expressing the cloned human α_{1A}-adrenoceptor. Concentration-dependent inhibitions of the response to NA (2 μM; the approximate $[A]_{80}$ for NA) were obtained without any effect upon basal levels. (Thus, evidence for endogenous agonist stimulation or antagonist 'negative efficacy' was not apparent). In each case, the profile of inhibition was apparently monophasic, with all antagonists, at high concentrations, inhibiting the response to NA by 100%. Affinity estimates made from these studies are summarized in Table 2. In each individual experiment, an E/[A] curve to NA was constructed to estimate a $p[A]_{50}$, which was then used with the Hill slope (n_H) and $p[B]_{50}$ to estimate antagonist affinity (pK_b). All computations were made by use of the Leff and Dougall (1993) modification of the Cheng and Prusoff (1973) equation. In most cases, the value of n_H for the antagonist inhibition curve did not differ significantly from that for the agonist E/[A] curve. Such a result would be expected for a competitive, reversible antagonist interacting under equilibrium conditions.

Data from InsPs accumulation studies in Tables 1–4 and in Figures 1 and 2 were taken from experiments in which a 10 min stimulation with agonist was used. However, separate experiments showed that affinity estimates for prazosin and RS-17053 in CHO-K1 cells expressing the cloned human α_{1A}-adrenoceptor were independent of exposure time to NA. Exposure times tested were 0.5, 1, 2, 5, 10 and 30 min (data not shown). In other experiments, inhibition curves for RS-17053 and prazosin were generated in CHO-K1 cells expressing the cloned human α_{1A}-adrenoceptor versus responses to a single concentration of two imidazoline agonists, A-61603 (0.1 μM) or oxymetazoline (0.3 μM). Preliminary results indicated that affinity estimates (pK_b) obtained were agonist-independent and did not differ significantly from those obtained versus NA (prazosin: $pK_b = 8.46$ versus oxymetazoline, 8.71 versus A-

Table 1 Potency ($p[A]_{50}$) and intrinsic activity (i.a.) estimates for agonists: [^{3}H]-InsPs accumulation in CHO-K1 cells expressing cloned human α_{1A}-, α_{1B}- and α_{1D}- adrenoceptors

Agonist		n	*InsPs* $p[A]_{50}$	*InsPs* *i.a.*	*Slope* n_H	*Fold stimulation* (× basal)
NA	α_{1A}	12	6.63±0.05	1.0	0.96±0.05	10.0±1.6
NA	α_{1B}	8	6.53±0.07	1.0	1.02±0.09	3.72±0.43
NA	α_{1D}	5	7.73±0.10	1.0	0.96±0.11	4.14±0.46
Phenylephrine	α_{1A}	3	6.31±0.13	0.99±0.03		
Methoxamine	α_{1A}	4	5.83±0.08	0.84±0.07		
SDZ NVI 085	α_{1A}	4	5.90±0.03	0.78±0.05		
Amidephrine	α_{1A}	4	6.27±0.12	1.01±0.03		
NS-49	α_{1A}	3	6.28±0.04	0.72±0.13		
A-61603	α_{1A}	5	8.37±0.05	1.09±0.05		
Oxymetazoline	α_{1A}	3	8.12±0.18	0.58±0.04		

Intrinsic activity estimates relative to maximal response produced by NA (i.a. = 1.0). Values are means±s.e.mean from number of determinations shown (*n*).

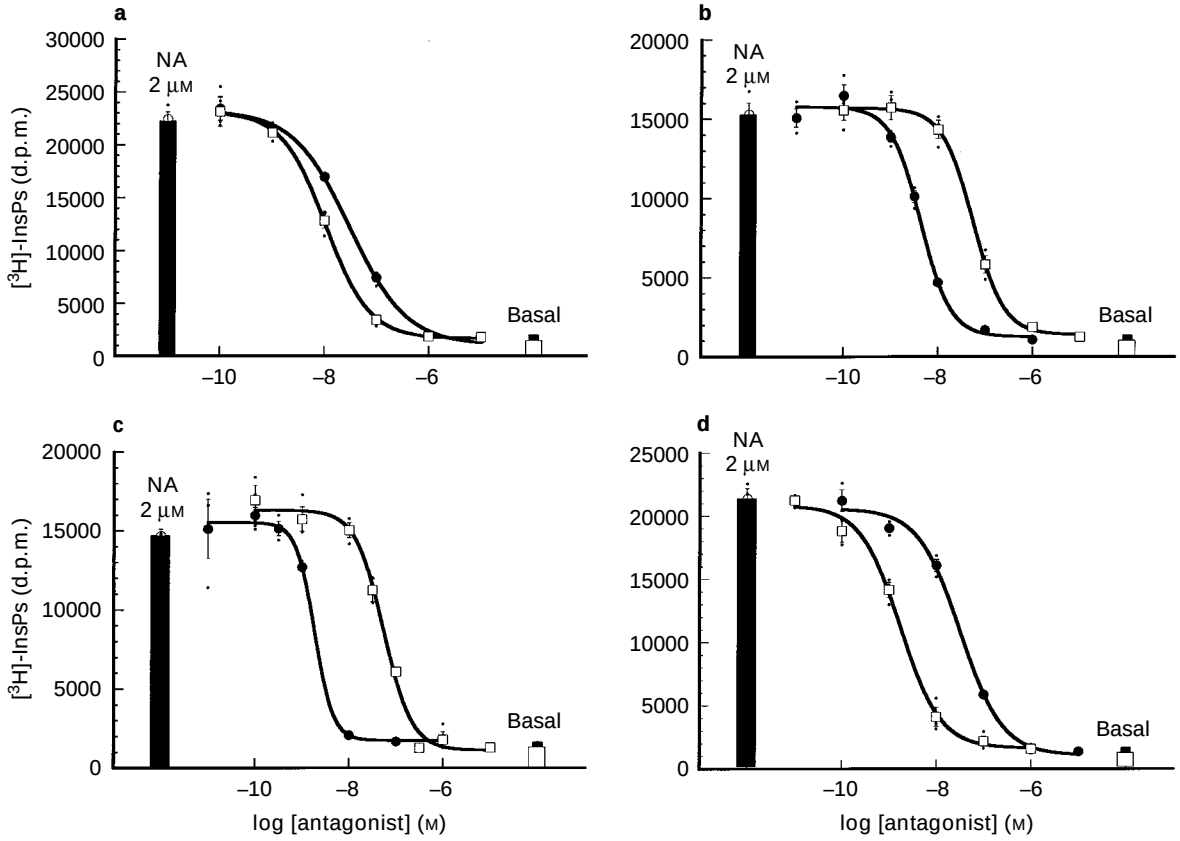

Figure 2 Inhibition curves for selected antagonists versus noradrenaline (NA)-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing the human cloned α_{1A}-adrenoceptor. Responses were obtained to a single concentration (2 μM; approximate $[A]_{80}$) of NA alone or after equilibration with increasing concentrations of antagonist (20 min equilibration, 10 min agonist stimulation). (a) Effects of (□) prazosin and (●) RS-17053; (b) effects of (●) WB 4101 and (□) 5-methyl-urapidil; (c) effects of (●) tamsulosin and (□) *S*-niguldipine; (d) effects of (□) Rec 15/2739 and (●) indoramin. Data shown are from representative single experiments performed in triplicate. Within-experiment means and standard deviations are shown (large symbols), as are individual data points, through which non-linear regressions were constructed. Each experiment was repeated, with number of experiments shown in Table 2. Inhibition curve slopes for tamsulosin and S-niguldipine (c) were noticeably steep ($n_H > 1$).

Table 2 Data from antagonist inhibition curves obtained against NA ($[A]_{80}$, 2 μM)-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human cloned α_{1A}-adrenoceptors

Antagonist	*α_{1A}-CHO InsPs* pK_b	*α_{1A}-CHO InsPs* n_H	n
Prazosin	8.73±0.02	1.2±0.1	3
RS-17053	8.24±0.06	1.3±0.1	3
WB 4101	8.84±0.13	1.3±0.1	4
5-Me-urapidil	8.10±0.03	1.2±0.1	4
S-niguldipine	8.38±0.30	3.1±1.0*	3
Tamsulosin	9.56±0.09	2.3±0.3*	3
Tamsulosin[a]	10.53±0.08	1.0±0.1	3
Rec 15/2739	9.39±0.03	1.4±0.2	3
Indoramin	8.39±0.04	1.1±0.2	3

pK_b estimated according to Leff & Dougall (1993): $K_b = IC_{50}/((2+([A]_{80}/[A]_{50})^{n_H})^{1/n_H}-1)$. Values are means± s.e.mean from number of determinations shown. *Hill slopes (n_H) significantly greater than 1 ($P<0.05$). [a]Obtained against 100 μM NA.

Table 3 Comparison of affinity estimates from radioligand binding and [^{3}H]-InsPs studies in α_{1A}-CHO-K1 cells with functional studies in isolated perfused kidney of rat (α_{1A}-adrenoceptors) and lower urinary tract tissues of man (α_{1L}-adrenoceptors

Antagonist	*α_{1A}-CHO binding* pK_i	*α_{1A} rat kidney* pA_2	*α_{1L} prostate* pA_2	*α_{1A}-CHO InsPs* PK_b
Prazosin	9.9±0.0	9.5	8.7±0.1	8.7±0.1
RS-17053	9.3±0.1	9.8	7.3±0.2	8.3±0.1
WB 4101	9.8±0.1	10.3	8.9±0.1	8.8±0.1
5-Me-urapidil	9.2±0.1	9.2	8.2±0.1	8.1±0.1
S-Niguldipine	9.9±0.2	10.5	7.5±0.5	8.2±0.3
Tamsulosin	10.4±0.2	10.0	10.4±0.1	10.5±0.1
Rec 15/2739	9.6±0.2	No data	9.2±0.2	9.4±0.1
Indoramin	8.4±0.1	No data	8.5±0.2	8.4±0.1

pK_b for prazosin and RS-17053 for [^{3}H]-InsPs is pA_2 estimated from Schild regression analysis. For tamsulosin affinity estimate for [^{3}H]-InsPs is pK_b obtained versus higher concentration of NA (100 μM). Values are means±s.e.mean ($n \geqslant 3$). Data for α_{1A}-adrenoceptors in rat kidney were from Blue *et al.* (1995) and for α_{1L}-adrenoceptor in lower urinary tract tissues from Ford *et al.* (1996).

61603, each from single study performed in triplicate. RS-17053: $pK_b = 8.16$ and 8.17, in independent triplicate studies versus oxymetazoline).

Competition radioligand binding studies in membrane homogenates of CHO-K1 cells expressing cloned human α_{1A}-adrenoceptors revealed a pharmacological profile typical for the classically defined α_{1A}-adrenoceptor (Hieble *et al.*, 1995). Saturation analysis yielded a pK_b estimate for [^{3}H]-prazosin of 9.92±0.01 and a B_{max} of 1.64±0.20 pmol mg^{-1} protein. Consistent with the pharmacology of the α_{1A}-adrenoceptor, prazosin, RS-17053 (Ford *et al.*, 1996), WB 4101, 5-methylurapidil, Rec 15/2739, tamsulosin and *S*-niguldipine all dis-

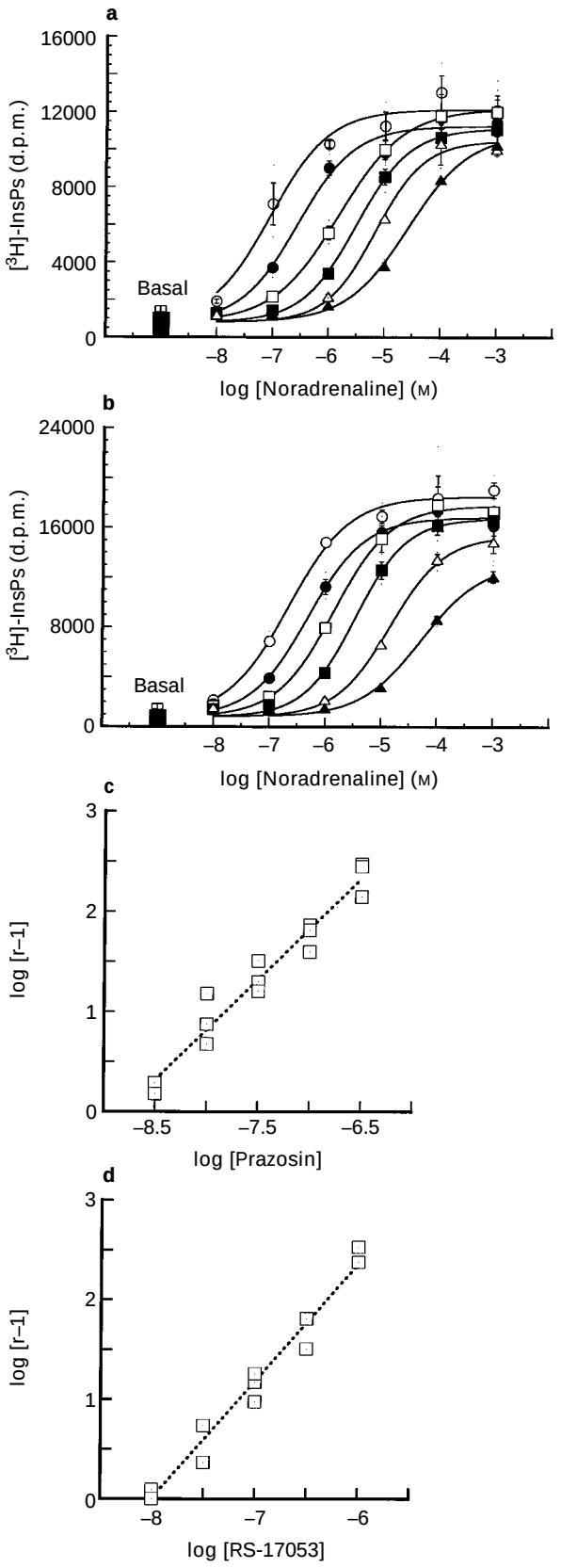

Figure 3 Schild regression analyses for prazosin and RS-17053 antagonism of NA-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human cloned α_{1A}-adrenoceptor. E/[A] curves were obtained to NA, alone (○) or after equilibration with different concentrations (●, 10 nM; □, 30 nM; ■, 100 nM; △, 300 nM; ▲, 1000 nM) of (a) prazosin and (b) RS-17053 (20 min equilibration, 10 min agonist stimulation). (a and b) E/[A] curves shown are from representative single experiments performed in triplicate. Within-experiment means and standard deviations are shown (large symbols), as are individual data points, through which non-linear regressions were constructed. Each experiment was performed on 3 separate occasions. (c and d) Schild regressions constructed with data from 3 series of E/[A] curves for each antagonist. For prazosin (c), linear regression results yielded a slope of 1.01 (95% CL 0.86–1.15), pA_2 estimate of 8.78, r^2 value of 0.95. For RS-17053 (d), linear regression results yielded slope of 1.17 (95% CL 1.03–1.32), pA_2 estimate of 8.03, r^2 value of 0.97.

played subnanomolar affinity ($pK_i > 9.0$) in competition studies, with indoramin giving a predictably lower affinity of 8.4 (see Table 3). These data are in excellent agreement with values from radioligand binding studies for the cloned human α_{1A}-adrenoceptor published previously (Forray *et al*., 1994; Testa *et al*., 1995; Tseng-Crank *et al*., 1995; Shibata *et al*., 1995). Similarly, antagonist affinity profiles (see Table 3) obtained versus [^{3}H]-prazosin in CHO-K1 cells stably expressing human α_{1B}- and α_{1D}-adrenoceptors ([^{3}H]-prazosin pK_D estimates of 10.35 ± 0.02 and 9.85 ± 0.03 and B_{max} values of 1.22 ± 0.13 and 1.17 ± 0.05 pmol mg^{-1} protein, respectively) were consistent with those published previously (Forray *et al*., 1994; Hieble *et al*., 1995).

Table 3 shows that the affinity for certain antagonists in [^{3}H]-inositol phosphates accumulation assays were lower than might be expected for an α_{1A}-adrenoceptor, as reflected by the comparison with competition binding affinity estimates. In order to test rigorously the validity of these functional affinity estimates, two key antagonists, prazosin and RS-17053, each displaying a 10 fold separation in affinity estimates from binding to function, were investigated further by using a more classical pharmacological approach. Complete E/[A] curves to NA were constructed alone and in the presence of several concentrations of antagonist (10–1000 nM). E/[A] curves from one such study, performed in triplicate, are shown in Figure 3. It can be seen that increasing concentrations of prazosin (a) and RS-17053 (b) caused a progressive, concentration-dependent rightward displacement of E/[A] curves to NA. Displacements were parallel, with no change in curve maxima, except at the highest concentration of RS-17053 (1000 nM). Transformation of the data from 3 separate experiments allowed construction of Schild regressions, which are also shown in Figure 3. In the case of prazosin, the slope of the regression was 1, which is fully consistent with competitive, reversible antagonism under equilibrium conditions. An affinity estimate (pK_b) of 8.82 ± 0.04 was generated. For RS-17053, the slope of the Schild regression (1.17) differed significantly from 1. Under these conditions, the pA_2 value of 8.0 cannot be assumed to reflect the equilibrium dissociation constant. However, if the highest concentration (1000 nM) of RS-17053 was excluded from the regression analysis (as it caused depression of E_m), the slope became statistically equivalent to 1 (at the 95% confidence level), and a pK_b estimate of 8.13 ± 0.05 was obtained. (A single point analysis using the excluded, 1000 nM concentration of RS-17053, yielded an 'apparent pA_2, assuming a regression slope of 1' of 8.3). Thus, in the case of both prazosin and RS-17053, affinity estimates made from the E/[A] curve method did not differ significantly from those calculated by the inhibition curve method. Therefore, the pK_b values obtained for other antagonists with marginally steep Hill slopes (WB 4101, 5-methylurapidil, Rec 15/2739 and indoramin, see Table 2) may be considered valid estimates of affinity.

However, in the case of tamsulosin and *S*-niguldipine inhibition curves were markedly steeper than the respective agonist E/[A] curves, rendering estimates of affinity difficult. Steep inhibition curves may result from inadequate equilibration, or alternatively, loss of antagonist due to degradation, binding to surfaces, or a specific removal process, or all three. Longer antagonist equilibration times (up to 120 min) were without effect on curve slope or affinity estimate, ruling out temporal disequilibrium (not shown). The possible loss of antagonist (which may become particularly critical at low concentrations) was investigated for tamsulosin, by two ap-

proaches. First, E/[A] curves to NA were constructed alone and in the presence of 3 concentrations of tamsulosin (1, 3 and 10 nM). Tamsulosin displaced E/[A] curves to NA in a parallel manner, with no change in maxima, but the displacements were not linearly related to concentration. The resulting Schild regression is shown in Figure 4b. As can be seen, from the data from two independent triplicate studies, a steep slope over the entire regression could be generated (slope = 1.72) although a linear relationship was barely justifiable. However, by examination of the two highest concentrations alone, a partial regression could be generated with a slope of 1 and an accordingly higher affinity estimate ($pA_2 = 10.1$) compared with a pK_b of 9.6 which was obtained by use of the inhibition curve method (versus NA, 2 μM).

The potential confounding influence of drug loss was investigated further by utilizing higher concentrations of tamsulosin, so as to saturate possible clearance mechanisms. To accommodate this, the inhibition curve design was modified by raising 50 fold the concentration of NA, to 100 μM. This approach improved the shape of the inhibition curves for tamsulosin (see Figure 4a), yielding a Hill slope of 1.03 ± 0.14 ($n = 3$): a value not different from that for the E/[A] curves to NA studied in the absence of tamsulosin. A higher affinity estimate (pK_b) was calculated for tamsulosin (10.53 ± 0.08) and this value was employed for correlation studies given below. Unfortunately, similar experiments with the lipophilic dihydropyridine, *S*-niguldipine, did not yield an unequivocal improvement in affinity estimate. Thus, the value given for this antagonist must be regarded as tentative.

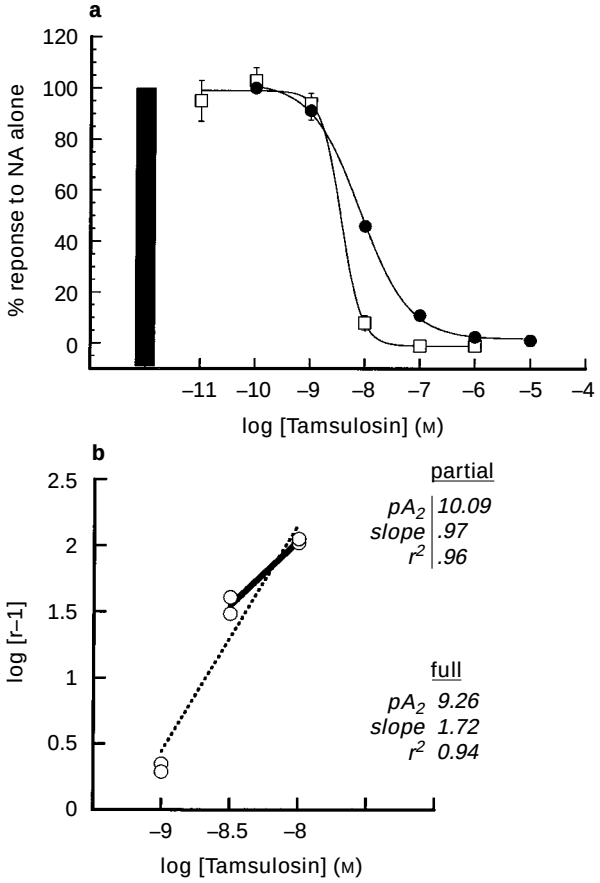

Figure 4 Further analysis of the antagonism by tamsulosin of NA-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human cloned α_{1A}-adrenoceptor. In (a), inhibition curves are shown for antagonism by tamsulosin of the [^{3}H]-InsPs accumulation response to two different concentrations of NA: 2 μM (□) and 100 μM (●). Hill slope (n_H) close to unity (−0.95) was observed only for the higher concentration of NA; n_H for 2 μM NA = −2.30. Affinity estimates (pK_b) were 9.42 for 2 μM NA and 10.56 for 100 μM NA and were obtained from this individual triplicate experiment which was performed on three separate occasions. (b) Schild regression plot for tamsulosin (1, 3 and 10 nM). Full regression is shown as dashed line ($pA_2 = 9.26$, slope = 1.72, $r^2 = 0.94$). A partial regression of the two highest concentrations is indicated by the solid line ($pA_2 = 10.09$, slope = 0.97, $r^2 = 0.96$). Each point represents concentration-ratio (r) data from a single experiment performed in triplicate.

Table 4 gives functional and binding results for the cloned human α_{1B}- and α_{1D}-adrenoceptors, which were expressed at densities similar to that for the α_{1A}-adrenoceptor. Similar affinity estimates for the same eight antagonists were obtained in both functional and radioligand binding assays. These data, illustrating the high affinity estimate for prazosin ($pK_b = 9.6$, for both receptors), are consistent with the contention that the lower affinity estimate for prazosin, and other key antagonists, obtained with the cloned human α_{1A}-adrenoceptor, did not arise simply as a result of methodological underestimation.

Figure 5 shows the relationship between affinity estimates from functional and radioligand binding experiments for the α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors. In each panel, the line of identity (y = x) is drawn (dotted line). In (a), it is clear that the relationship between affinity estimates for the α_{1A}-adrenoceptor in CHO cells was poor, with 5 antagonists obviously distant from the line of identity. The high sum of squares of affinity differences ($\Sigma(y-x)^2 = 7.67$) supports this inequality. In contrast, the correlation in (b), for InsPs studies (α_{1A}-adrenoceptor) versus functional affinity estimates made previously in human lower urinary tract (LUT) (Ford *et al*., 1996) was much improved ($\Sigma(y-x)^2 = 1.56$, compared with 13.18 for LUT versus data from radioligand binding studies). Comparison of LUT data with those obtained for InsPs responses for the α_{1B}- and α_{1D}-adrenoceptors in CHO cells revealed considerably lower similarity ($\Sigma(y-x)^2 = 6.02$ and 4.06, respectively). Figures 5(c) and (d) show the relationships between affinity estimates for InsPs versus binding for the α_{1B}- and α_{1D}-adrenoceptors in CHO cells, and in each case the degree of correlation was fairly good ($\Sigma(y-x)^2 = 1.79$ and 2.48, respectively).

Discussion

The α_{1A}-adrenoceptor is the most thoroughly characterized of the α_1-adrenoceptor family. The original definition, based on high affinity for WB 4101, 5-methylurapidil and *S*-niguldipine has been strengthened considerably by the addition of further α_{1A}-adrenoceptor-selective antagonists, some from new chemical series, such as SNAP 5089 (Wetzel *et al*., 1995), Rec 15/2739 (SB 216469) (Testa *et al*., 1995), KMD 3213 (Shibata *et al*., 1995), and RS 17053 (Ford *et al*., 1996). The high, subnanomolar affinity displayed by all of these antagonists, along with subnanomolar affinities of prazosin and tamsulosin, and relatively high affinity of indoramin, provides a pharmacolo-

Table 4 Affinity estimates for α-adrenoceptor antagonists from radioligand binding and [^{3}H]-InsPs studies in α_{1B}- and α_{1D}-CHO cells

Antagonist	*α_{1B}-CHO binding* pK_i	*α_{1B}-CHO InsPs* pK_b	*α_{1D}-CHO binding* pK_i	*α_{1D}-CHO InsPs* pK_b
Prazosin	9.9±0.0	9.63±0.03	9.9±0.1	9.61±0.30
RS-17053	8.0±0.1	7.63±0.08	7.8±0.1	7.28±0.09
WB 4101	9.0±0.1	8.61±0.06	9.6±0.1	9.03±0.05
5-Me-urapidil	7.7±0.1	7.62±0.01	8.0±0.1	8.21±0.14
Tamsulosin	9.6±0.1	9.40±0.18	10.1±0.1	9.80±0.27
S-niguldipine	7.7±0.1	6.55±0.07	7.4±0.1	6.71±0.22
Rec 15/2739	7.8±0.1	7.74±0.16	7.8±0.1	8.80±0.13
Indoramin	7.4±0.1	7.73±0.02	6.7±0.1	7.06±0.14

Values are means±s.e.mean from 3 separate triplicate determinations. Binding data for indoramin from Forray *et al*. (1994).

gical fingerprint that is unique for the 'classical' α_{1A}-adrenoceptor (Hieble *et al*., 1995).

In contrast, the definition of the α_{1L}-adrenoceptor has been less rigorously documented, although some recent studies have established characteristics in addition to the eponymous low affinity for prazosin (Holck *et al*., 1983; Flavahan & Van-Houtte, 1986), including relatively low affinities for WB 4101, 5-methylurapidil (Muramatsu, 1992), RS-17053 and the dihydropyridines, *S*-niguldipine and SNAP 5089 (Ford *et al*., 1996). Important as these α_{1A}-/α_{1L}-adrenoceptor discriminating antagonists are, equally critical to the definition of the α_{1L}-adrenoceptor are the non-discriminating antagonists, tamsulosin, Rec 15/2739 and indoramin. The latter three antagonists offer internal validation that an assay system is not merely underestimating affinities of antagonists through methodological oversight. It is only with the use of this broad range of high affinity probes that a clear characterization of the α_{1L}-adrenoceptor and its separation from other subtypes can be achieved.

Agonists, on the other hand, appear to offer little utility in discrimination of the α_{1L}-adrenoceptor from the α_{1A}-adrenoceptor. While it is becoming clear that agonists from diverse chemical series, including phenethylamines (e.g., amidephrine, NS 49, methoxamine, SDZ NVI 085) and imidazolines (e.g., oxymetazoline, A-61603), offer degrees of selectivity for the α_{1A}-adrenoceptor over the α_{1B}- and α_{1D}-adrenoceptors, in terms of affinity and/or efficacy (see Eltze & Boer, 1992; Minneman *et al*., 1994; Blue *et al*., 1995; Knepper *et al*., 1995), data have not emerged to support agonist discrimination between the α_{1L}-adrenoceptor and the α_{1A}-adrenoceptor. Consequently, it is not surprising that the profile of agonist potency and intrinsic activity found in the current study are entirely consistent with α_{1A}-adrenoceptor activities in recombinant and native functional assay systems (see Eltze & Boer, 1992; Minneman *et al*., 1994; Blue *et al*., 1995; Knepper *et al*., 1995).

The surprising finding from the present study relates solely to the equilibrium dissociation constants estimated for the eight competitive α_1-adrenoceptor antagonists studied. As can be seen from Table 3 and Figure 5, a different profile of affinity estimates was obtained in InsPs studies compared with binding studies when the cloned human α_{1A}-adrenoceptor was used. Prazosin, WB 4101, 5-methylurapidil, RS-17053 and *S*-niguldipine showed supranananomolar affinity estimates (pK_b), that were 10 to 40 fold lower than in membrane binding studies. However, affinity estimates were not merely 'frameshifted' down as tamsulosin, indoramin and Rec 15/2739 yielded si-

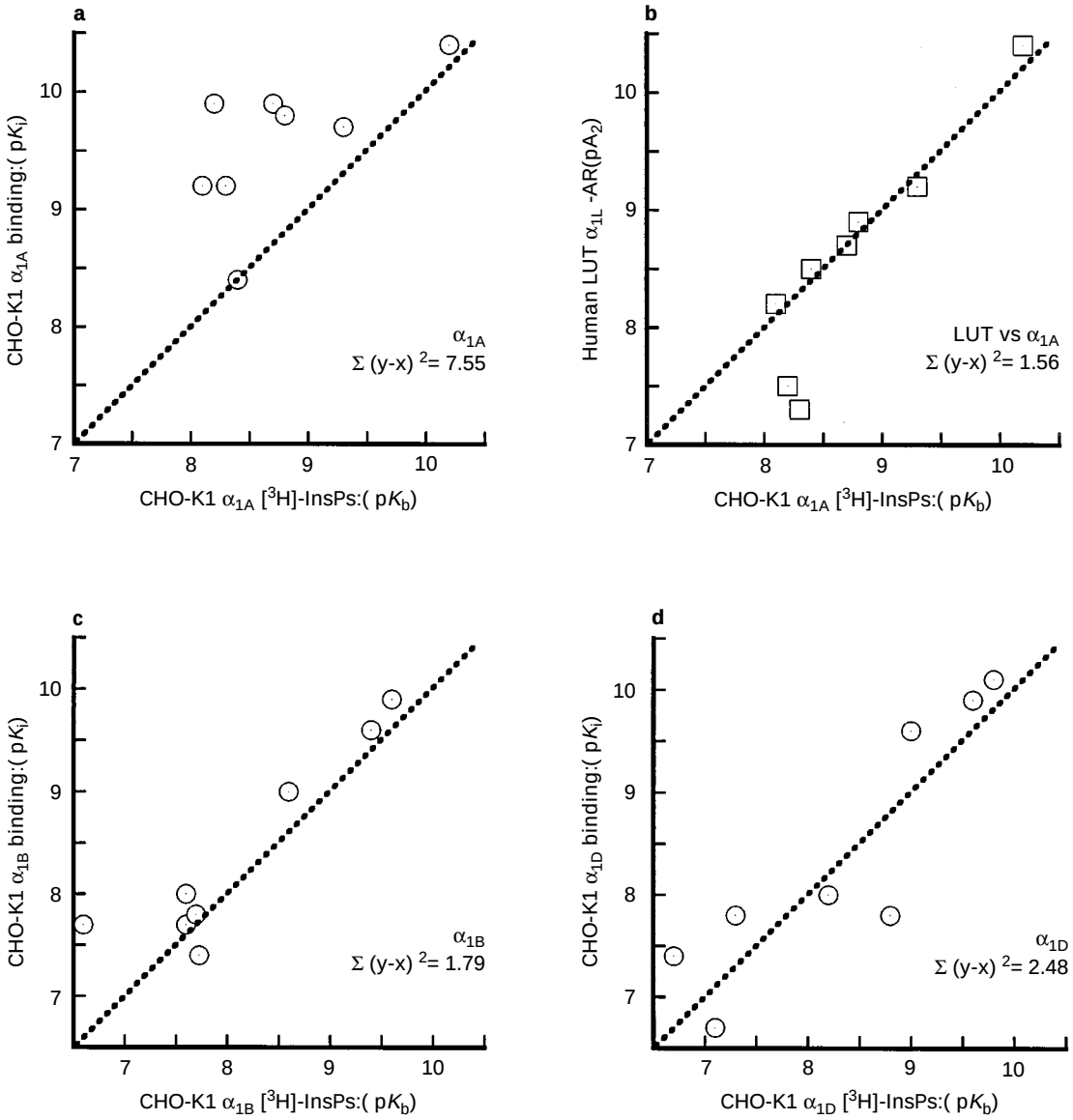

Figure 5 Correlation plots showing relationship of affinity estimates from [^{3}H]-InsPs accumulation studies for selected antagonists in CHO-K1 cells expressing human cloned α_1-adrenoceptors. In each plot, dashed line indicates line of identity ($y=x$). $\Sigma\ (y=x)^2$ is the sum of squares of differences in affinity estimates for each plot, and describes, in relative terms, how different are two data sets. Data are from Table 3 (a, b) and Table 4 (c, d).

milar, high affinity estimates in both binding and InsPs assays. Inspection of data obtained for the same antagonists in human LUT tissues (periurethral, prostatic and bladder neck smooth muscles; Ford *et al*., 1996) revealed an interesting similarity. Functional affinity estimates for prazosin, WB 4101, 5-methylurapidil, RS-17053 and *S*-niguldipine were, likewise, all lower in human LUT tissues compared with binding estimates at cloned human α_{1A}-adrenoceptors. In addition, the same three antagonists, tamsulosin, indoramin and Rec 15/2739 gave affinity estimates that differed little between the functional and binding assays. The similarities between affinity estimates for InsPs studies (α_{1A}-adrenoceptor) versus functional affinity estimates human LUT (Ford *et al*., 1996) are well demonstrated by the reduction in the sum of squares data. Clearly, the α_{1A}-adrenoceptor functional data agree best with LUT data, as those obtained for InsPs responses for the α_{1B}- and α_{1D}-adrenoceptors revealed considerably lower similarity. This would be consistent with the very minor presence of mRNA and radioligand binding evidence for the latter two adrenoceptors in human LUT tissues (Forray *et al*., 1994; Faure *et al*., 1994).

Thus, the eight antagonists studied may be divided into two groups: those which gave affinity estimates for the α_{1A}-adrenoceptor in the InsPs assay which were not substantially different from those found in membrane homogenate binding assays (tamsulosin, Rec 15/2739 and indoramin; affinity constants differ by $\leqslant 0.4$ log units); and those which show a separation in affinity estimates between the two assays (prazosin, RS-17053, WB 4101, 5-methylurapidil and *S*-niguldipine; affinity constants differ by $\geqslant 0.9$ log units).

The validity of the estimates of affinity for the antagonists was obviously of concern. However, in most cases the Hill slopes for inhibition of NA stimulations did not differ significantly from those for the stimulation E/[A] curves in the absence of antagonists, consistent with the attainment of equilibrium conditions. Furthermore, the Schild regressions performed for prazosin and RS-17053 validated the inhibition curve design, as identical affinity determinations for these critical antagonists were generated. Finally, it may be expected that any artefactual underestimate of affinity (due perhaps to methodological reasons) would have resulted in a 'frame-shift' of estimates, for α_{1B}- and α_{1D}-adrenoceptors, as well as α_{1A}-adrenoceptors. This clearly did not happen.

In those cases where Hill slopes were greater than 1, further examination yielded satisfactory resolution for tamsulosin, indicating significant removal of this antagonist at low concentrations ($\leqslant 1$ nM). However, in the case of *S*-niguldipine attempts to obtain equilibrium data were not totally successful, due presumably to the extreme lipophilicity and chemical instability of this antagonist. Thus, the affinity constant estimated for *S*-niguldipine may be considered to be an underestimate. This latter contention could feasibly be extended to previous studies with dihydropyridine antagonists. (For example, studies made in the human LUT (Ford *et al*., 1996) where rigorous estimation of affinity for *S*-niguldipine and SNAP 5089 was precluded). Likewise, a low affinity estimate ($pA_2 = 7.3$) for RS-17053 was obtained in human LUT, most probably because of its intrinsically high lipophilicity. Such studies, in which segments of smooth muscle were used in static tissue baths, provide a large 'tissue sink' for lipophilic compounds which is difficult (if not impossible) to saturate over a desirable concentration range. Reduced tissue mass, as in the current study, is likely to lead, therefore, to more reliable affinity estimates. Interestingly, in a recent study (Lachnit *et al*., 1997), the ability of these 'problematic' ligands to characterize α_{1A}-adrenoceptors (i.e., display high affinity) in a tissue bath assay was tested and confirmed, in strips of caudal artery from rat.

The behaviour of other ligands used in the present study did not deviate significantly from that expected for simple competition under equilibrium conditions. The excellent agreement of affinity estimates for RS-17053 and prazosin when studied by use of inhibition curve versus parallel displacement (Schild analysis) designs supports the validity and concentration independence of the derived values. It is also worthy of mention that while lipophilic ligands may be prone to removal by non-specific processes (e.g., tissue 'sink'), RS-17053 and Rec 15/2739, each of which are highly lipophilic, differed considerably in their abilities to discriminate between α_{1A}- and putative α_{1L}-adrenoceptors.

As implied above, experimental conditions can affect thermodynamic equilibria and perturb affinity estimates (see Kenakin, 1993). In general terms, therefore, it is not surprising that affinity estimates from a functional assay (with whole cells, responding to agonist stimulation, in isotonic media at 37°C) failed to match perfectly affinity determinations from radioligand binding (membrane homogenates, in which antagonist displacement was measured, low ionic strength media, room temperature). What is surprising is that affinity estimates were modified differentially, producing a profile consistent with the putative α_{1L}-adrenoceptor. These data furnish the distinct potential that the unique pharmacology of the α_{1L}-adrenoceptor, observed in many functional tissue studies, is explicable by this putative receptor being a conformational state of the α_{1A}-adrenoceptor. If true, this obviates the quest for the α_{1L}-adrenoceptor gene.

Clearly, it would be interesting to identify the factor or factors that cause this apparent α_{1A}- to α_{1L}-adrenoceptor 'switch'. It is possible that the intact cellular conditions required for the InsPs studies are such that radioligand binding studies performed in whole cells, rather than homogenates, may yield similar affinity estimates: assuming that available radioligands permit such studies (see below). However, it is apparent from the close correlation between function and binding for the α_{1B}- and α_{1D}-adrenoceptors, that such a result would have to be specific for the α_{1A}-adrenoceptor.

In this regard, recent data from the human cloned α_1-adrenoceptor subtypes expressed stably in CHO-K1 cells indicate that the conditions under which radioligand binding studies are performed are able to influence their pharmacological properties (Williams *et al*., 1996). Accordingly, in the recent studies, affinity estimates for the same antagonists as used in the present study were observed to be typical for the 'classical' α_{1A}-adrenoceptor when performed in membrane homogenates (Tris buffer, 20°C), and yet became consistent with those described for the α_{1L}-adrenoceptor when performed in whole cells (Ham's buffer, 37°C). No such affinity state pleiotropism was observed for α_{1B}- and α_{1D}-adrenoceptors under these differing conditions.

It is also worth mentioning that, to date, a clear α_{1A}-adrenoceptor pharmacological profile has not been described in human tissues, except from radioligand binding studies. Thus, for the human α_{1A}-adrenoceptor, the pharmacological phenotype of the α_{1L}-adrenoceptor may represent the more stable whole-cell 'ground-state' of the receptor and may be more generally observed.

The present observations promote the following conclusions. First, it would appear that the use of homogenate radioligand binding studies in recombinant systems may not predict the behaviour of pharmacological probes in whole cells, tissue segments, and more importantly, in intact animals. Therefore, the selection of drug candidates without adequate functional analysis may be misleading, as properties may differ from those observed in an intact system. Secondly, and more generally, what has been observed here for the α_{1A}-adrenoceptor may occur in other systems where tissue- and assay-specific pharmacological properties have been described. One example is the M_3 muscarinic cholinoceptor, where, within the same species, several ligands (e.g., zamifenacin, *p*-fluorohexahydro-siladifenidol) are able to display tissue-specific affinity estimates in functional assays (Eglen *et al*., 1994). Interestingly, radioligand binding data do not appear to support these functional discrepancies.

In summary, it appears that the human α_{1A}-adrenoceptor gene product can display the pharmacological properties of the α_{1L}-adrenoceptor (as defined in human lower urinary tract

tissues) as well as those characteristic of the α_{1A}-adrenoceptor. Why these two pharmacological phenotypes occur and whether this truly explains the α_{1L}-adrenoceptor pharmacology observed in many native tissues, requires further investigation.

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